



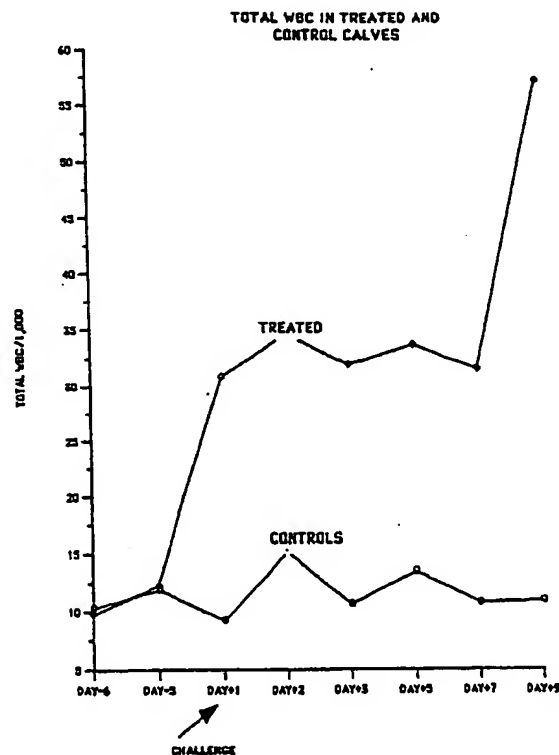
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(54) Title: COMPOSITIONS AND METHOD FOR TREATING OR PREVENTING INFECTIONS IN ANIMALS

## (57) Abstract

Compositions and method for treating or preventing bacterial infections such as mastitis in animals, particularly bovine animals, which comprises administering an effective amount of granulocyte colony stimulating factor (G-CSF), are disclosed. The G-CSF may be naturally derived, or alternatively, the G-CSF and genetically engineered variants of G-CSF may be the expression products of genetically engineered prokaryotic or eukaryotic host cells.



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COMPOSITIONS AND METHOD FOR TREATING  
OR PREVENTING INFECTIONS IN ANIMALS

This application is a continuation-in-part of  
5 Ser. No. 193,857 filed May 13, 1988 hereby incorporated  
by reference.

Field Of The Invention

The present invention is directed to the use  
10 of granulocyte colony stimulating factor (G-CSF) to  
treat or prevent infections in animals. More  
specifically, the invention is directed to the use of  
G-CSF, in particular, human G-CSF (hG-CSF) or  
bovine G-CSF (bG-CSF), in treating or preventing  
15 infections. The source of the G-CSF may be naturally  
derived or may be derived from genetically engineered  
prokaryotic or eukaryotic host cells containing  
recombinant plasmid or viral DNA vectors carrying the  
human or bovine G-CSF gene, or genetically engineered  
20 variants of human or bovine G-CSF genes, or synthetic  
human or bovine G-CSF genes. The present invention is  
also directed to DNA gene segments, biologically  
functional recombinant plasmids and viral DNA vectors,  
and prokaryotic and eukaryotic host cells containing  
25 such recombinant plasmids and vectors, all of which  
contain a bovine G-CSF gene or a genetically engineered  
variant of a bovine G-CSF gene.

BACKGROUND OF THE INVENTION

30

Infections in animals result in losses of  
billions of dollars per year in the meat and dairy  
industries. Although antibiotic therapy is now used for  
animal infections with some success, huge losses  
35 persist. Examples of such infections are mastitis in  
cows, and shipping fever in cattle.

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Bovine AnimalsA. Mastitis

The most costly problem in dairying today is mastitis. Mastitis is defined as an inflammation of the mammary gland. It may affect any mammal, for example cows, ewes, and goats, but bovine mastitis is of the greatest economic importance. Bovine mastitis is an infection of the udder of ruminants such as cows, mainly caused by gram positive and gram negative bacteria and especially in cows in intensive milk producing units. The bacterial infection results in the inflammation of the mammary gland (i.e. teats and udder). The disease is particularly troublesome and of considerable economic importance because the pathogen is readily transferred from one animal to another during the milking process. Some of the main pathogenic microorganisms causing bovine mastitis are Staphylococcus aureus, Streptococcus agalactiae, Streptococcus uberis, Streptococcus dysgalactiae, Escherichia coli, Aerobacter aerogenes, Klebsiella pneumoniae, and Pseudomonas aeruginosa. See also Bovine Mastitis, edited by Glenys Bloomfield, V&O Publications 1987, hereby incorporated by reference. These microorganisms invade the udder through the teat canal and produce inflammation of the milk-producing tissue causing the formation of scar tissue which, once formed, may cause a permanent reduction in the cow's milk production. An infection can also alter the composition, quantity, appearance and quality of the milk.

There are a variety of forms or types of bovine mastitis, with varying severity and symptomatology, including the following:

- (1) Udder infection: The invasion of the udder cavity by microorganisms that multiply within the gland and cause inflammation;

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(2) Nonclinical or subclinical mastitis; A form of mastitis in which there is no swelling of the gland or observable abnormality of the milk, although there are changes in the milk that can be detected by special tests. This type of mastitis is by far the most prevalent and causes the greatest overall loss in most herds. It often is referred to as "hidden" mastitis;

(3) Clinical mastitis; A form of mastitis in which the abnormal conditions of the udder and secretion are observable. Mild clinical mastitis involves changes in the milk such as flakes, clots, and a watery or unusual appearance. Heat and sensitiveness of the udder are slight or absent, but there may be signs of swelling. Severe clinical mastitis involves a sudden onset with swelling of the infected quarter which is hot, hard and sensitive. The milk appears abnormal and milk production drops. Sometimes, in addition to the local effects in the udder, the cow herself becomes sick. There are signs of fever, rapid pulse, depression, weakness and loss of appetite. The combination of these conditions often is referred to as acute systemic mastitis, because not only the udder, but the whole animal is affected; and

(4) Chronic mastitis; A form of mastitis caused by a persistent udder infection that exists most of the time in the nonclinical form but occasionally can develop into an active clinical form. After these "flare-ups" the nonclinical form usually returns temporarily. (See generally Current Concepts of Bovine Mastitis, published by The National Mastitis Council, Inc., 2nd Ed. 1978 at p.5.)

Mastitis continues to cause large economic losses to the dairy industry. Mastitis affects the profitability of a herd in a number of ways, both directly and indirectly, including: (1) loss of milk production; (2) higher culling rates of infected cows;

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(3) decreased value of milk; (4) discarded milk following antibiotic treatment; (5) veterinary costs (antibiotics and veterinary visits); and (6) deaths. (Bovine Mastitis, Glenys Bloomfield, supra, at p.33.)

5 An estimate of the yearly cost in the United States is \$1 billion in milk alone. Treatment and culling costs raise this estimate of total costs to about \$1.5 to \$2 billion per year in the United States, and this represents the effect of mastitis on only 5% of the  
10 world dairy population. (Current Concepts of Bovine Mastitis, supra, at p.7.) While world figures are sketchy, projections from U.S.D.A. figures reveal a world-wide loss figure exceeding \$20 billion annually.

Effective compounds to be used in the treatment  
15 or prevention of bovine mastitis should give the following results: (1) most or all of the above pathogens should be susceptible to the compound when the pathogen is in milk and other udder fluids; (2) the therapeutic effect should be relatively quick; (3) no  
20 significant irritation should be caused to the teats or udder of the cow, either by the active or other ingredients of the composition; and (4) the active compound should not stay in the milk for a period much in excess of the time required for the therapeutic activity  
25 so as to minimize the loss of milk, which has to be discarded as long as a foreign compound is present. There are other requirements for such a composition for the treatment of bovine mastitis but the above four criteria are some of the most important ones.

30 Antibiotic therapy has been a major component of mastitis control strategy. Table 1 summarizes a number of the antibiotics used in the treatment of mastitis:

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Table 1

Antibacterial agents used  
in the treatment of mastitis

	Class	Compounds
5	1. <u>Beta-lactam antibiotics</u>	
	Penicillins	Ampicillin Cloxacillin Hetacillin Nafcillin Penicillin G (benzyl penicillin) Procaine penicillin
10		
	Cephalosporins	Cefoperazone ** Cefuroxime * Cefalonium * Cefapirin Cefoxazole * Cefracetrile *
15		
	2. <u>Aminoglycoside antibiotics</u>	Framycetin Neomycin Novobiocin Streptomycin
20	3. <u>Macrolide antibiotics</u>	Erythromycin
	4. <u>Tetracyclines</u>	Chlortetracycline Oxytetracycline
	5. <u>Polypeptide antibiotics</u>	Polymyxin B
	* First generation; ** third generation	
25	<u>Bovine Mastitis, supra, at p.70.</u>	

Antibiotic treatment for mastitis is usually given by means of intramammary infusions, either in lactating cows when clinical mastitis is detected, or at drying off (dry cow therapy). (Bovine Mastitis, supra, at p.69.) In cases where severe clinical disease is present, antibiotics must be given parenterally (intramammary infusions are ineffective because of blockage of the ducts). (Ibid.)

35

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The early hopes that antibiotics would allow complete control of the disease have not been realized. None of the above mentioned antibiotics utilized thus far has been entirely satisfactory.

5 Additionally, it has been found to be very desirable to replace antibiotic treatment with treatment by non-antibiotic chemo-therapeutic drug compounds, for the following reasons:

(1) Antibiotics effective in human medicine  
10 should not be utilized in veterinary medicine, in order not to build up strain resistance of bacteria appearing in human diseases;

(2) Antibiotics should be reserved for such diseases for which no chemo-therapeutic drug compound  
15 would be available, as it has been proved that bacterial strains build up resistance to an antibiotic after extended use of such antibiotic; and

(3) Staphylococcus aureus, one of the above-noted pathogens, has already built up a resistance  
20 against most of the antibiotics utilized in the treatment of bovine mastitis.

One such method for treatment by a non-antibiotic chemo-therapeutic drug compound is described in U.S. Patent 4,610,993 which claims a method for  
25 treating animals for bovine mastitis with an effective amount of at least one pyridine-N-oxide disulfide compound. Another method by the same inventors is described in U.S. Patent 4,401,666 which claims a method for treating animals for bovine mastitis with an  
30 effective amount of at least one metallic salt of pyridine 2-thione-N-oxide.

Despite these several published methods, it remains very important to find cost-effective methods utilizing non-antibiotic compounds which would  
35 substantially overcome the drawbacks of antibiotics used thus far and yet would be effective in treating and preventing mastitis.



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### B. Shipping Fever

Another common disease affecting the cattle industry is shipping fever (bovine respiratory disease). Respiratory diseases continue to be the major cause of disease loss in beef cattle. In a year-long study of diseases of 407,000 yearling feedlot cattle, respiratory tract diseases were responsible for about 3/4 of the clinical diagnoses and about 2/3 of the necropsy diagnoses.

10           The term "shipping fever" is used to describe the respiratory disease complex observed in cattle 6 months of age or older after shipment either into feedlots or onto pasture. The stresses of weaning, castration, dehorning, fasting, overcrowding, exposure  
15 to infectious agents, diet changes, environmental temperature extremes, and other stressors combined with viral, bacterial, mycoplasmal, and/or chlamydial infections contribute to the shipping fever complex. Mixing calves from different farms and/or salebarns  
20 greatly facilitates exposure to infectious agents. Population mixing may be a more important predisposing factor to shipping fever than stressors, although disease can occur without mixing and stressors usually dramatically worsen respiratory disease. Attempts to  
25 reduce stress by weaning, castrating, dehorning, etc. and acclimating cattle to new diets days or weeks prior to shipment are sometimes successful (but may not be cost-effective) in reducing the incidence of shipping fever. Vaccination against some of the infectious  
30 agents involved in shipping fever is sometimes helpful, but vaccines are available and efficacious for only a few of the agents known to be involved in the disease complex.

It is generally recognized that the ultimate  
35 cause of death in most cases of shipping fever is a bacterial (usually pasteurella) pneumonia.

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Pasteurella haemolytica, particularly type 1A, is the most common bacterium isolated from cases of respiratory disease in North America. Attempts to experimentally reproduce bacterial pneumonia in cattle are usually  
5: unsuccessful without severe stress and predisposing damage to the respiratory tract. It is generally believed that during times of stress, viruses, mycoplasma, and/or chlamydia most often provide the initial damage to the respiratory tract which  
10: predisposes to severe bacterial infection and disease.

A typical clinical respiratory disease outbreak usually begins within hours or days of the cattle's arrival at the feedlot. Recently shipped cattle in the 300 to 500 pound weight range commonly  
15: have a 20 to 80% morbidity and 1 to 10% mortality, or more, to respiratory tract disease. When the serum of cattle is analyzed for a four-fold antibody rise (seroconversion) and the respiratory tract and its secretions subjected to microbiologic isolations, a  
20: myriad of etiologic agents can be identified. Many animals, those sick and those apparently healthy, can be shown to have undergone infection by one or more agents (respiratory tract disease is probably seldom due to only one infectious agent). Although bovine respiratory  
25: disease complex is recognized clinically in the feedlot after arrival, the infections giving rise to clinical disease probably start at the salebarns, where cattle are first assembled from different farms. See also Bovine Respiratory Disease, Loan, R. W. Texas A & M  
30: University Press, 1984, hereby incorporated by reference.

#### Porcine Animals

Respiratory diseases, particularly pneumonia cost the swine industry hundreds of millions of dollars  
35: annually. It is the No. 1 problem of the growing-finishing period which accounts for half of the pig's

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life. Some of the common pathogens involved with pneumonia are Pasteurella multocida, Mycoplasma hyopneumonia, Haemophilus (Actinobacillus) pleuropneumonia, Streptococcus suis, Salmonella cholersuis, Bordetella bronchiseptica, pseudorabies virus, and swine influenza virus.

Haemophilus Actinobacillus pleuropneumoniae is a major cause of pneumonia in pigs. The disease is distributed worldwide and is one of the most economically important diseases of swine. In 1985 respiratory diseases in pigs were estimated to have resulted in a \$208 million loss to producers. Pleuropneumonia is the most feared respiratory disease because vaccines fail to prevent infections or prevent the occurrence of persistently infected carrier animals.

The economic impact of an acute outbreak of pleuropneumonia in an unexposed herd is apparent with death losses ranging from 0.4% to 24% and morbidity being between 8.5% and 40%. Treatment of the acute disease has relied on the use of antibiotics. The routine use of antibiotics is rapidly being limited due to public concerns and governmental pressures.

Detailed histomorphologic and bacteriologic studies of porcine pleuropneumonia reveal that i) early in the disease large numbers of neutrophils localize at the sight of the infection; ii) there is a relationship between the ability of the pigs to restrict the infection to the lung, thus preventing bacteremia, and survival; and iii) experimental depletion of circulating neutrophils results in a more severe disease.

### Equine Animals

The first two weeks of life (neonatal period) is a time when severe illness or death losses occur in foals due to infection (septicemia). Neonatal septicemia

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represents significant losses to the equine industry. In septicemic foals the neutrophil count is commonly very low (neutropenia) and this is one reason for the fatality rate of 60-75% with sepsis in the neonatal foal.

- 5               Problems secondary to gram negative sepsis such as bone and joint damage, enteritis, omphalophlebitis, respiratory disease and poor development also account for significant losses in the equine industry. Immunoglobulin levels have not correlated well with protection from
- 10 infection with 20% of septicemic foals having double the recommended level of IgG (400 mg/dl). Even with the advent of intensive care of the critically ill septic foal the survival rate is only 25-40%. Neutrophil function has been determined to be substantially reduced in foals with
- 15 colostrum deprivation. This combined with the frequent development of neutropenia with sepsis in the neonatal foal are important reasons for the high mortality and perhaps for the initiation of infection.

20 Granulocyte Colony Stimulating Factor

- Granulocyte colony stimulating factor (G-CSF) is one of several glycoprotein growth factors known as colony stimulating factors (CSFs) because they support the proliferation of haemopoietic progenitor cells.
- 25 G-CSF stimulates the proliferation of specific bone marrow precursor cells and their differentiation into granulocytes. It is distinguished from other CSFs by its ability to both stimulate neutrophilic granulocyte colony formation in semi-solid agar and to induce terminal
- 30 differentiation of murine myelomonocytic leukemic cells in vitro. Granulocyte Colony-Stimulating Factor is a potent stimulus for neutrophil proliferation and maturation in vivo (Cohen et al., Proc. Natl. Acad. Sci. 1987; 84: 2484-2488). G-CSF is also capable of inducing
- 35 functional activation or "priming" of mature neutrophils in vitro (Weisbart, R.H., Gasson, C.G., and D.W. Golde.

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Annals of Internal Medicine 1989; 110:297-303). G-CSF has been shown to prime human granulocytes, and enhance superoxide release stimulated by the chemotactic peptide, N-formyl-methionyl-leucyl-phenylalanine (S. Kitagawa, et al., Biochem. Biophys. Res. Commun. 1987; 144:1143-1146, and C.F. Nathan, Blood 1989; 74:301-306), and activate human neutrophil IgA mediated phagocytosis (Weisbart, R.H., et al., Nature 1988; 332: 647-649).

Neutrophils are a critical component of host defense mechanisms against bacterial and fungal infections. G-CSF is capable of inducing an increase in the absolute number of circulating neutrophils and enhances neutrophil function.

The cDNA cloning and expression of recombinant human G-CSF has been described, and it has been confirmed that the recombinant G-CSF exhibits most, if not all, of the biological properties of the native molecule (Souza, L. et al. Science 232, 61-65 (1986)). Sequence analysis of the cDNA and genomic DNA clones has allowed the deduction of the amino acid sequence and reveals that the protein is 204 amino acids long with a signal sequence of 30 amino acids. The mature protein is 174 amino acids long and possesses no potential N-linked glycosylation sites but several possible sites for O-linked glycosylation.

The cloning and expression of cDNA encoding human G-CSF has been described by two groups (Nagata, S. et al., Nature 319, 415-418 (1986); Souza, L. M. et al., Science 232, 61-65 (1986)). The first report of a G-CSF cDNA clone suggested that the mature protein was 177 amino acids in length. The authors reported that they had also identified a cDNA clone for G-CSF that coded for a protein that lacked a stretch of three amino acids. This shorter form of G-CSF cDNA expresses the expected G-CSF activity. The second report describes a cDNA sequence identical to this short form and makes no

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mention of other variants. Since these authors confirmed that the short cDNA expresses G-CSF with the expected profile of biological activity, it is probable that this is the important form of G-CSF and that the longer form  
5 is either a minor splicing variant or the result of a cloning artifact.

Matsumoto et al., in Infection and Immunity, Vol. 55, No. 11, p. 2715 (1987) discuss the protective effect of human G-CSF on microbial infection in  
10 neutropenic mice.

The following patent publications relate to G-CSF: WO-A-8703689, assigned to Kirin/Amgen describes hybridomas producing monoclonal antibodies specific for human G-CSF and their use in the purification of G-CSF;  
15 WO-A-8702060, assigned to Biogen, discloses human G-CSF like polypeptides and methods of producing them; U.S. Patent 4,810,643 assigned to Amgen, discloses human G-CSF like polypeptides, sequences encoding them and methods of their production; and WO-A-8604605 and WO-A-8604506, both  
20 assigned to Chugai Seiyaku Kabushiki Kaisha, disclose a gene encoding human G-CSF and infection inhibitors containing human G-CSF.

It is an object of the subject invention to provide an improved method of treating and preventing  
25 infections in animals.

It is a further object of the subject invention to provide a method of treating and preventing mastitis and shipping fever in bovine animals.

It is a further object of the subject invention  
30 to provide a method of treating infections in animals without build up of strain resistance of bacteria.

A still further object of the invention is to provide a purified and isolated polypeptide having part or all of the primary structural confirmation and one or more  
35 of the biological properties of naturally occurring bovine G-CSF, and DNA sequences encoding such bovine G-CSF.

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Other objects, features and characteristics of the present invention will become apparent upon consideration of the following description and the appended claims.

5

#### Summary Of The Invention

The present invention provides DNA sequences, biologically functional recombinant plasmids and viral  
10 DNA vectors, and prokaryotic and eukaryotic host cells containing such recombinant plasmids and vectors, all of which contain a bovine G-CSF gene or a genetically engineered variant of a bovine G-CSF gene. The invention also provides polypeptides encoded by the bovine G-CSF  
15 gene or variants thereof. A method for treating or preventing infections in animals is also disclosed.

Novel DNA sequences of the invention include sequences useful in securing expression in prokaryotic or eukaryotic host cells of polypeptide products having  
20 at least a part of the primary structural conformation and one or more of the biological properties of naturally occurring bovine granulocyte colony stimulating factor. DNA sequences of the invention are specifically seen to comprise: (a) the DNA sequence of  
25 the coding region of the mature protein, set forth in Figure 2a or its complimentary strand; (b) a DNA sequence which hybridizes (under hybridization conditions such as illustrated herein or more stringent conditions) to the DNA sequence in Figure 2a or to  
30 fragments thereof; and (c) a DNA sequence which, but for the degeneracy of the genetic code, would hybridize to the DNA sequence in Figure 2a. Specifically comprehended in part (b) are DNA sequences encoding allelic variant forms of bovine G-CSF and/or encoding  
35 other mammalian species of granulocyte colony stimulating factor. Specifically comprehended by part

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(c) are manufactured DNA sequences encoding bovine G-CSF, fragments of bovine G-CSF and analogs of bovine G-CSF with DNA sequences incorporating codons facilitating translation of messenger RNA in microbial  
5 hosts. Such manufactured sequences may readily be constructed according to the methods of Alton, et al., PCT published application WO 83/04053.

A further embodiment of the invention relates to synthetic genes designed to allow for expression of  
10 bovine G-CSF in E. coli.

The invention also comprises a method for purifying G-CSF.

Also comprehended by the invention are pharmaceutical compositions comprising effective amounts  
15 of polypeptide products of the invention together with suitable diluents, adjuvants and/or carriers useful in animal therapy.

The subject invention also relates to a method for treating and preventing infections in animals such  
20 as bovine animals, by administering a therapeutically effective treating or preventing amount of granulocyte colony stimulating factor, advantageously G-CSF derived from the gene of the animal to be treated. In addition, the invention relates to a method of treating or  
25 preventing mastitis or shipping fever by administering a therapeutically effective treating or preventing amount of granulocyte colony stimulating factor.

#### Brief Description of the Drawings

30

Figure 1 shows the restriction map of bovine G-CSF;

Figure 2a illustrates the coding region of the mature protein of bovine G-CSF.

35

Figure 2b is the amino acid sequence of the mature protein;



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Figure 3 is the genomic sequence of the human G-CSF;

Figure 4 is the DNA sequence of a first bovine G-CSF synthetic gene (bG-CSF dna);

5        Figure 5 illustrates the oligos used to construct the subunits of a further bovine G-CSF synthetic gene (bG-CSF dna4);

Figure 6 shows the two subunits of the bovine G-CSF synthetic gene bG-CSF dna4;

10       Figure 7 shows the amino acids coded by the bovine G-CSF synthetic gene bG-CSF dna4;

Figures 8-12 are graphic representations of the results obtained in Example 4 which relates to treatment of cows infected with Pasteurella Hemolytica,  
15   with G-CSF;

Figures 13-17 show the results obtained in Example 5 which relate to the use of G-CSF to control bovine respiratory disease;

Figures 18-20 show results obtained in  
20   Example 6 which examine the effect of G-CSF on respiratory disease;

Figures 21-23 are graphic representations of results obtained in Example 7 which relate to infection of cows with Klebsiella Pneumonia; and

25       Figures 24-26 are graphic representations of the results obtained in Example 8 where cows having coliform mastitis were treated with G-CSF.

Figures 27-29 are graphic representations of the results obtained in Examples 10-12.

30

### Detailed Description Of The Invention

A novel method for treating or preventing infections in animals has been discovered. Surprisingly  
35   it has been found that G-CSF is effective in a method of treating or preventing infections in animals, particularly mammals.

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A variety of infections afflicting bovine animals, poultry, swine, horses, as well as dogs and cats, are treatable with G-CSF by the method of the subject invention.

5           Mastitis in bovine animals can be effectively treated with G-CSF. By "bovine animals" is meant a cow or ox. The subject invention also relates to treating or preventing diseases such as shipping fever in cattle by administration of G-CSF. "Mastitis" is an  
10 inflammatory disease of the mammary gland caused by a variety of pathogenic microorganisms, primarily gram-positive and gram-negative bacteria. A veterinarian of ordinary skill can readily determine whether an animal exhibits a mastitis infection. In one  
15 embodiment, the present invention relates to a method of treating or preventing infections such as mastitis in a non-human animal comprising administering a composition which comprises an effective amount of G-CSF.

The subject invention also relates to treating  
20 or preventing diseases in horses by administration of G-CSF. For example, neonatal septicemia can be treated with G-CSF.

Increasing the number of circulating neutrophils in pigs during an acute outbreak of  
25 pleuropneumonia provides protection from infection and reduces the severity of the disease and the likelihood of persistent infections. G-CSF can be used to treat or prevent porcine infections such as pleuropneumonia.

By G-CSF is meant one of the hematopoietic  
30 growth factors known as granulocyte colony stimulating factors. The biological activities of G-CSFs include: stimulating the differentiation of a small number of progenitor "stem cells" into the variety of blood cell lines, stimulating the proliferation of those blood cell  
35 lines and stimulating the ultimate differentiation of mature blood cells from those lines. The preferred

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sources of the G-CSF polypeptide for treating or preventing mastitis are human and bovine, and may be naturally-derived or the product of genetically engineered host cells containing a DNA sequence encoding

5 G-CSF.

The DNA encoding the G-CSF gene is a genomic DNA sequence, a cDNA sequence or a manufactured (or synthetic) DNA sequence which is expressed in a prokaryotic or eukaryotic host cell as a polypeptide

10 having part or all of the primary structural conformation and one or more of the biological properties of naturally-occurring G-CSF. A biologically functional plasmid or viral DNA vector containing a DNA sequence encoding G-CSF may be used to transform or

15 transfect a prokaryotic or eukaryotic host cell to produce cell lines expressing the G-CSF polypeptide, glycosylated or unglycosylated.

The various forms of human G-CSF, including their preparation and purification, useful in a method

20 for treating or preventing mastitis are described in detail in U.S. Patent 4,810,643, having a common assignee, which is hereby incorporated by reference. U.S. Patent 4,810,643 describes and claims novel gene segments, biologically functional recombinant plasmids

25 and viral DNA vectors and prokaryotic and eukaryotic host cells, which contain a G-CSF gene or a genetically engineered variant of a G-CSF gene. The host cells express biologically active G-CSF or a genetically engineered variant of G-CSF.

30 This application describes the isolation and characterization of a bovine G-CSF gene and in particular describes and claims novel gene segments, biologically functional recombinant plasmids and viral DNA vectors, and prokaryotic and eukaryotic host cells,

35 which contain a bovine G-CSF gene or a genetically engineered variant of a bovine G-CSF gene. The host

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cells transformed or transfected with the recombinant plasmids or viral DNA vectors express biologically active bovine G-CSF or a genetically engineered variant of bovine G-CSF. The protein expressed is purified  
5 using the method described below or methods well known to those skilled in the art.

DNA sequences coding for all or a part of bovine G-CSF are provided. Such sequences include: the incorporation of codons "preferred" for expression by  
10 selected non-mammalian hosts (e.g. E. coli preferred codons, see Nucleic Acids Res. 1986 vol.14 (13) pp 5125-5143); the provision of sites for cleavage by restriction endonuclease enzymes; the provision of DNA sequences which reduce or eliminate secondary structure  
15 interactions which inhibit transcription and/or translation; and the provision of additional initial, terminal or intermediate DNA sequences which facilitate incorporation into expression vectors. The DNA sequences of the invention also include sequences having  
20 an optimized ribosome binding site, and sequences which enhance transcription, translation, and/or secretion of the protein product.

The present invention also provides DNA sequences coding for microbial expression of polypeptide  
25 analogs or derivatives of bovine G-CSF which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues (i.e., deletion analogs containing less than all of the residues specified for bovine G-CSF; substitution analogs, wherein  
30 one or more residues specified are replaced by other residues; and in addition, analogs wherein one or more amino acid residues are added to a terminal or medial portion of the polypeptide) and which share some or all the properties of naturally-occurring forms.

35 Also comprehended by the present invention is that class of polypeptide coded for by portions of the DNA



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complement to the top strand bovine cDNA or genomic DNA sequences of Figures 2a or 3 herein, i.e., "complementary inverted proteins" as described by Tramontano, et al., Nucleic Acids Res., 12, 5049-5059 (1984).

5           The present invention relates to purified and isolated polypeptide products having part or all of the primary structural conformation (i.e., continuous sequence of amino acid residues) and one or more of the biological properties (e.g., immunological properties and in vitro  
10 biological activity) and physical properties (e.g., molecular weight) of naturally-occurring bovine G-CSF including allelic variants thereof. These polypeptide are also characterized by being the product of chemical  
15 synthetic procedures or of procaryotic or eukaryotic host expression (e.g., by bacterial, yeast, higher plant, insect and mammalian cells in culture) of exogenous DNA sequences obtained by genomic or cDNA cloning or by gene synthesis. The products of typical yeast (e.g.,  
20 Saccaromyces cerevisiae) or prokaryote [e.g., [Escherichia coli (E. coli)] host cells are free of association with any mammalian proteins. Depending upon the host employed, polypeptide of the invention is glycosylated with mammalian or other eukaryotic carbohydrates or is non-glycosylated. Polypeptides of the invention may also  
25 include an initial methionine amino acid residue (at position -1).

          In addition to naturally-occurring allelic forms of bovine G-CSF, the present invention also embraces other bovine G-CSF products such as polypeptide  
30 analogs of bovine G-CSF and fragments of bovine G-CSF. All such forms of bovine G-CSF may be useful in the method for treating or preventing infections such as mastitis in animals. Following the procedures of the published application by Alton, et al. (WO/83/04053),  
35 hereby incorporated by reference, one can readily design and manufacture genes coding for microbial expression of

- 20 -

polypeptides having primary conformations which differ from that herein specified for, in terms of the identity or location of one or more residues (e.g., substitutions, terminal and intermediate additions and deletions).

5 Alternately, modifications of genomic and cDNA genes are readily accomplished by well-known site-directed mutagenesis techniques which generate analogs and derivatives of bovine G-CSF. Such products share at least one of the biological properties of bovine G-CSF

10 but may differ in others. As examples, products of the invention include those which are foreshortened (e.g., by deletions); or those which are more stable to hydrolysis (and, therefore, have more pronounced or longer lasting effects than naturally-occurring); or which have been

15 altered to delete one (or more) potential site(s) for n-linked or o-linked glycosylation (which result in higher activities for yeast-produced products); or which have one or more cysteine residues deleted or replaced (for example, by alanine or serine residues) and are more

20 easily isolated in active form from microbial systems; or which have one or more tyrosine residues replaced by phenylalanine and bind more or less readily to G-CSF receptors on target cells. Also comprehended are polypeptide fragments duplicating only part of the

25 continuous amino acid sequence or secondary conformations with bovine G-CSF, which fragments possess one activity of (e.g., receptor binding) and not others (e.g., colony growth stimulating activity).

According to another aspect of the present

30 invention, the DNA sequence described herein which encodes bovine G-CSF polypeptides is valuable for the information which it provides concerning the amino acid sequence of this bovine protein (and similar mammalian proteins) which has heretofore been unavailable. The

35 DNA sequences are also conspicuously valuable as products useful in effecting the large scale microbial

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synthesis of bovine G-CSF by a variety of recombinant techniques. Put another way, DNA sequences provided by the invention are useful in generating new and useful viral and plasmid DNA vectors, new and useful transformed and transfected prokaryotic and eukaryotic host cells (including bacterial, yeast, and mammalian cells grown in culture), and new and useful methods for cultured growth of such microbial host cells capable of expression of bovine G-CSF and G-CSF variants or analogs. DNA sequences of the invention are also conspicuously suitable materials for use as labelled probes in isolating bovine G-CSF and related protein encoding cDNA and genomic DNA sequences of other mammalian species. DNA sequences are also useful in various alternative methods of protein synthesis (e.g., in insect cells) or in genetic therapy in mammals. DNA sequences of the invention are useful in developing transgenic mammalian species which may serve as eukaryotic "hosts" for production of bovine G-CSF and bovine G-CSF products in quantity. (See generally Palmiter, et al., Science, 22(4625), 809-814 (1983))

Of applicability to bovine G-CSF fragments and polypeptide analogs of the invention are reports of the immunological activity of synthetic peptides which substantially duplicate the amino acid sequence extant in naturally-occurring proteins, glycoproteins and nucleoproteins. More specifically, relatively low molecular weight polypeptides have been shown to participate in immune reactions which are similar in duration and extent to the immune reactions of physiologically significant proteins such as viral antigens, polypeptide hormones, and the like. Included among the immune reactions of such polypeptides is the provocation of the formation of specific antibodies in immunologically active animals. (See, e.g., Lerner, et al., Cell, 23: 309-310 (1981); Ross, et al., Nature,



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- 294: 654-656 (1981); Walter, et al., Proc. Natl. Acad. Sci. (USA), 77: 5197-5200 (1980); Lerner, et al., Proc. Natl. Acad. Sci. (USA), 78: 4882-4886 (1981); Wong, et al., Proc. Natl. Acad. Sci. (USA), 78: 7412-7416 (1981); Green, et al., Cell, 28: 477-587 (1982); Nigg, et al., Proc. Natl. Acad. Sci. (USA), 79: 5322-5326 (1982); Baron, et al., Cell, 28: 395-404 (1982); Dreesman, et al., Nature, 295: 183-190 (1982); and Lerner, Scientific American, 248 (2): 66-74 (1983)). See, also, Kaiser, et al. Science, 223: 249-255 (1984) relating to biological and immunological activities of synthetic peptides which approximately share secondary structures of peptide hormones but may not share their primary structural conformation.

15 All of the above mentioned forms, fragments, variants and analogs of bovine G-CSF may be useful in the method of treating or preventing infections such as mastitis in animals as described herein.

In another embodiment of the invention, one or more additional colony stimulating factors are administered to the infected animal with G-CSF, egs. GM-CSF, M-CSF and multi-CSF (IL-3). The CSFs are administered together or separately. In a further embodiment, animal infections are treated by administering G-CSF with one or more of: the interferons (advantageously  $\alpha$ -interferon), IL2, and TNF or, with traditional antibiotics such as penicillins, cephalosporins, and amino-glycosides.

This application also describes pharmaceutical compositions of G-CSF in a pharmaceutically acceptable carrier. These compositions may be administered intravascularly, intraperitoneally, subcutaneously, intramuscularly, or by intramammary infusion using forms known to the pharmaceutical art. For intravascular, intraperitoneal, subcutaneous, intramuscular or intramammary administration, active drug components may

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be combined with a suitable carrier such as water, saline, aqueous dextrose, and the like. Regardless of the route of administration selected, the compositions of the present invention are formulated into

5 pharmaceutically acceptable dosage forms by conventional methods known to those skilled in the art. Advantageous formulations are described in commonly owned Ser. No. 285,159 hereby incorporated by reference. In one embodiment, sustained release formulations are used.

10 In another embodiment, G-CSF treatment is used in a prophylactic manner. G-CSF may be used as a prophylactic therapy to augment the host defense of animals who are at risk for acquiring a bacterial, yeast, or fungal infection. For example, G-CSF can be  
15 used as a prophylactic therapy in normal human patients at risk of acquiring pneumonia (e.g. nosocomial pneumonia), surgical wound infections, urinary tract infections, and intestinal infections. The term "normal" as used herein means an animal which has normal  
20 immune function and normal white blood cell count and differential. A description of the various nosocomial infections which the prophylactic therapy of the subject invention can be used against are stated in Nelson et al., J. Critical Illness, p12-24 (April 1988) hereby  
25 incorporated by reference. As a prophylactic G-CSF is administered typically 0-3 days before the event (e.g. surgery, vascular cannulation, catheter insertion, intubation) where there is a risk of infection. G-CSF is administered subcutaneously or intraventricularly  
30 daily in the dose range of 1 to 20  $\mu\text{g}/\text{kg}$ , more advantageously, 3 to 15  $\mu\text{g}/\text{kg}$ . A person skilled in the art will know how to adjust the dose.

In another embodiment, G-CSF is used to treat a patient immediately or soon, advantageously within  
35 eight hours, most advantageously within four hours after the patient has received a traumatic injury (e.g. human

- 24 -

or animal bite, gunshot or knife wound or other puncture wound) requiring medical or surgical treatment. G-CSF is administered prior to detection (e.g. by standard microbiological or histological examination of the patient's tissues or body fluids) or clinical manifestation and symptoms (e.g. purulent exudate, chills, fever) of infection.

Cattle are treated prophylactically prior to shipping or other occurrences which may debilitate the cattle, in order to boost and prime their capacity to fight off infections. Administration of the G-CSF can be made at the time the cattle are processed, i.e. vaccinated, branded, etc. Treatment with G-CSF can also be made during dry cow therapy and/or just before a cow gives birth in order to reduce the likelihood of post partum intrauterine infections, and of mastitis during the early stages of lactation. See Kehrli et al., Am. J. Vet. Res., 50, No.2, 207 (1989) for a description of bovine neutrophil function during the periparturient period. Conventionally, there is no treatment with antibiotics just before birth because of residues which would appear in the cows milk making it unfit for use.

Several variables will be taken into account by the ordinary artisan in determining the concentration of G-CSF in the therapeutic formulations and dosages to be administered. Variables include administration route and condition of the animal.

### 30 Purification of G-CSF

G-CSF is produced in an insoluble form in E. coli. The purification procedure described below was developed to assure a monomeric, oxidized product. In order to accomplish this, the protein is solubilized in the presence of a denaturant and allowed to oxidize in

- 25 -

the presence of this denaturant. The oxidized protein is then removed from the denaturant and purified to homogeneity. The following narrative describes the procedure for purifying human G-CSF with differences noted for the purification of bovine G-CSF.

#### Cell Lysis

350 g of frozen cell paste is combined with 2 - 3 liters of 1 mM dithiothreitol (DTT) and thoroughly dispersed. The cell suspension is then passed through a Gaulin homogenizer for a sufficient number of cycles to achieve greater than 95% breakage. The slurry is cooled to  $5 \pm 3^{\circ}\text{C}$  prior to homogenization and to below  $18^{\circ}\text{C}$  between cycles through the homogenizer. The homogenization slurry is centrifuged to recover the pellet, which contains >95% of the G-CSF originally present in the cell paste. (The bovine G-CSF breakage mixture contains no DTT).

20

#### Extraction

The homogenized pellet is next dispersed in about 3 liters of 1% deoxycholate (DOC), 5 mM DTT, 5 mM EDTA, 50 mM Tris, pH 9. This suspension is stirred at  $15-20^{\circ}\text{C}$  for 30-40 minutes and then centrifuged. (This DOC extraction step is not used for bovine G-CSF). The pellet is then resuspended in approximately 3 liters of cold water and recentrifuged. The G-CSF is recovered in the pellet with an overall yield of >80%. As an alternative to deoxycholate, another bile salt or non-ionic detergent is used.

35

#### Solubilization and Oxidation

The final pellet obtained from the above extractions is suspended in approximately 2.5 liters of

- 26 -

2% Sarkosyl, 40 mM Tris (pH 8.0) and allowed to solubilize at 15 - 20°C for 30 minutes. Copper sulfate is added to a final concentration of 40  $\mu$ M, and the mixture is allowed to stir at least 12 hours at 15-20°C. Approximately 70% of the G-CSF oxidizes into the correct monomeric form during this procedure.

#### Removal of Sarkosyl

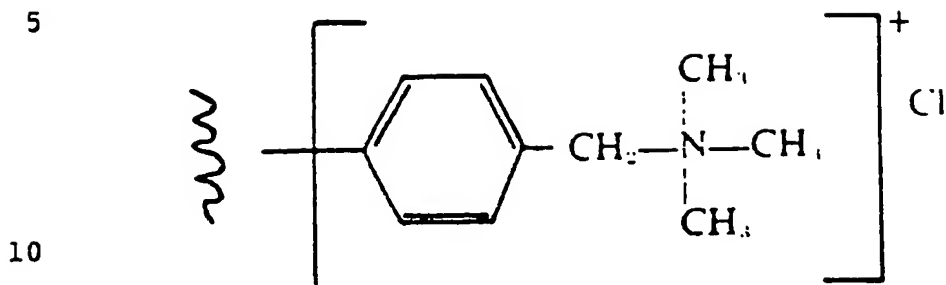
The resultant solubilized protein mixture is centrifuged to remove insoluble debris and then diluted four-fold with 13.3 mM Tris, pH 7.7. About 2000 g of Dowex equilibrated in 20 mM Tris, pH 7.7 is then added. Dowex is an ion exchange resin. See Dowex: Ion Exchange, The Dow Chemical Company, Midland, MI (1988) hereby incorporated by reference. Advantageously, Dowex 1X4 20-50 mesh Type 1 is used, which is a strong base anion exchange resin, chloride form. This resin is a 4% crosslinked styrene-divinylbenzene polymer matrix with quaternary ammonium functional groups which serve as sites for ion exchange. "Type 1" means that the ammonium group is composed of a nitrogen atom bonded to a polymeric benzyl group, which is attached to the matrix, and three methyl groups. The mesh size is measured as wet resin on U.S. standard screen. The ammonium group is positively charged; this functionality will exchange its associated chloride ion for other negatively charged ionic species in a reversible reaction. Dowex 2 resin is a Type II resin in which one of the methyl groups is replaced by an ethanol group.

30

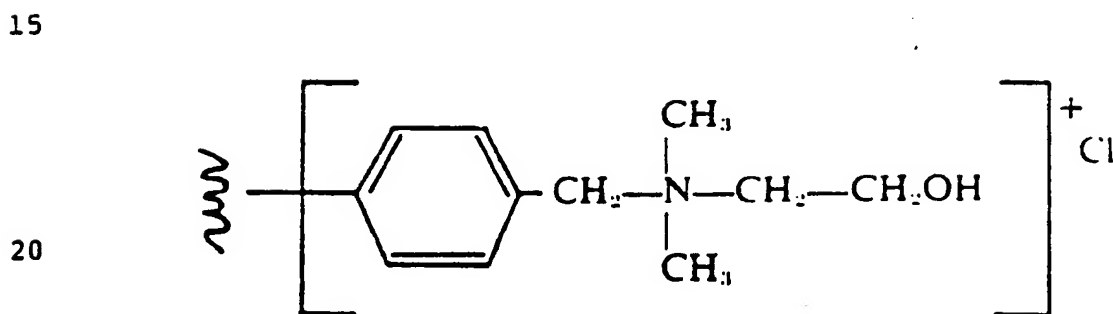
35

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## Type I



## Type II



25

Type I and Type II resins differ primarily in their affinities for the hydroxide ion relative to other anions, and in chemical stability. Type II resins are more efficiently converted to the hydroxide form than Type I resins, but the Type I resins are inherently more stable chemically, especially in the hydroxide form.

35

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The quaternary ammonium anion-exchange resins are highly ionized and can be used over the entire pH range. They are also capable of salt-splitting reactions, which convert a neutral salt to the  
5 corresponding base.

Other ion exchange resins which have strong anion exchange properties wherein the charge is inaccessible to the proteins of interest are alternatives to the use of Dowex in the subject  
10 invention.

This mixture is allowed to stir at least one hour at 15-20°C to remove Sarkosyl (N-Lauroylsarcosine sodium salt) (Sigma) from the protein solution, and then the Dowex is filtered out. Greater than 80% of the  
15 correctly oxidized form of G-CSF can be recovered. (The bovine G-CSF is processed at 2-8°C during this step).

#### Anion Exchange Chromatography (This Step Not Used For Bovine G-CSF)

20

The resultant protein solution is applied to a DEAE-cellulose (Whatman DE-52 or equivalent) column equilibrated in 20 mM Tris, pH 7.7. The column is washed with 20 mM Tris, pH 7.7, until the absorbance at  
25 280 nm of the eluant is approximately zero. The G-CSF is then eluted off the column with 20 mM Tris, 40 mM NaCl, pH 7.7.

#### Cation Exchange Chromatography

30

The eluent from the DEAE column is adjusted to pH 5.4 with 50% acetic acid and diluted at least two-fold with 5 mM sodium acetate, pH 5.4. The solution is loaded onto a CM-Sepharose, fast flow column  
35 equilibrated in 20 mM sodium acetate, pH 5.4. After loading, the column is washed with 20 mM sodium acetate,

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pH 5.4, until the absorbance at 280 nm is approximately zero. The G-CSF is then eluted with 20 mM sodium acetate, 37.5 mM NaCl, pH 5.4. (The bovine G-CSF is eluted off at 150 mM NaCl in the same buffer).

5

\* \* \*

The following examples are presented by way of illustration of the invention and are specifically directed to procedures carried out prior to identification of bovine G-CSF genomic and cDNA clones, to procedures resulting in such identification, and to the sequencing, development of expression systems based on genomic, cDNA and manufactured (or synthetic) genes and verification of expression of bovine G-CSF and analog products in such systems. The method of isolating the bovine G-CSF gene described below can also be used to isolate other animal G-CSF genes, which in turn can be used in producing other animal G-CSF. In addition, the examples illustrate methods for treating or preventing infections in animals, comprising administering an effective amount of G-CSF.

#### EXAMPLE 1

25

##### Screening a Genomic Library for the Bovine G-CSF Gene

In this example, a cDNA clone encoding human G-CSF as described in commonly owned U.S. Patent 4810643 was used to screen for a genomic clone containing a bovine G-CSF gene. A phage (Charon 28) bovine genomic library prepared according to the procedure of Woychick, et. al., Nucleic Acid Research 10(22): 7197-7210 (1982) and obtained from J. Bloom and F. Rothman was plated out on E. coli strain K802 and screened using a nick translated probe consisting of a human G-CSF cDNA



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fragment isolated from HgiAI to StuI. A total of approximately  $1.2 \times 10^6$  phage were plated on 12, 15 cm petri dishes, plaque lifted and hybridized to probe using the procedures described in Maniatus et al. Molecular Cloning, A Laboratory Manual (1982). A total of 7 positive clones were observed. Three clones yielding the strongest signals upon autoradiography in a secondary screening were grown in 1 liter cultures and phage DNA was prepared as described in Maniatus et al. Molecular Cloning, A Laboratory Manual (1982). This DNA was mapped by restriction enzyme digestion and Southern blotting using the radiolabeled HgiAI to StuI probe. The mapping results showed that a BamHI fragment of about 3000 bases contained the entire G-CSF region. DNA from clone 2 was digested with BamHI to release an approximately 3000 bp bovine G-CSF containing fragment which was subsequently subcloned into pUC9 and further mapped by restriction endonuclease digests and Southern blotting.

A restriction endonuclease map (approximately 3.0 kb) of genomic DNA containing the bovine G-CSF gene is shown in Figure 1. The sequence for the entire coding region of the mature bovine G-CSF was determined by subcloning fragments into M13 and sequencing them by the dideoxy method Sanger et al., Proc. Natl. Acad. Sci. USA, 74:5463 (1977). Sequences were confirmed or extended by utilizing internal primers off of the same clones. The sequence for the coding region was deduced by direct comparison with the human genomic G-CSF sequence (Figure 3) and is shown in Figure 2a. Splice juncture sites and amino terminal processing of the protein were assumed to occur at the same places as the human G-CSF. The DNA sequence codes for a mature protein of the same length as the human G-CSF (174 amino acids) and the proteins are 82% homologous.

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## EXAMPLE 2

Construction of Synthetic Bovine G-CSF Genes  
and Expression of Bovine G-CSF

5

This example relates to preparation of manufactured genes encoding bovine G-CSF and including E. coli preference codons, and to expression of bovine G-CSF.

10

Synthetic genes were designed to allow for the expression of bovine granulocyte colony stimulating factor (bG-CSF) in E. coli [bG-CSF dna (Figure 4) and bG-CSF dna4 (Figures 5-7)]. bG-CSF is 174 amino acids in length and is 82% homologous to the human form of G-CSF (174 a.a.). The bovine protein contains five more charged residues (arg and glu) than does hG-CSF and is therefore believed to be more hydrophilic.

15

The genes bG-CSF dna (Figure 4) and bG-CSF dna4 (Figures 5-7) were designed with maximum bias for E. coli codon preference. For gene bG-CSF dna, in addition to the coding sequence, an initiation ATG, a ribosome binding site, two termination codons, and a 5' XbaI and 3' BamH1 restriction sites were included. The gene bG-CSF dna4 was also designed to have minimum secondary interactions and sufficient unique restriction sites for subunit assembly and gene manipulation. Unique BamH1 and PstI sites were incorporated at positions identical to those found in the hG-CSF gene noted in commonly owned U.S. Patent 4810643. This allows for generation of unique human/bovine hybrid genes and their protein products.

25

30

The gene was designed as two subunits (Subunit I XbaI-HindIII, and Subunit II HindIII-EcoR1) for cloning into sequencing/expression vectors (Figure 6). Subunit I contains a short leader sequence with a XbaI cloning end and the ribosome binding site (RBS).

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Subunit II contains a pair of redundant stop codons and the EcoRI cloning end.

Briefly stated, the protocol employed was generally as set out in the disclosure of co-owned  
5 Alton, et al., PCT Publication No. WO83/04053, which is incorporated by reference herein. The genes were designed for initial assembly of component oligonucleotides (Figure 5) into multiple duplexes which, in turn, were assembled into two discrete  
10 sections (Figure 6). These sections were designed for ready amplification and, upon removal from the amplification system, could be assembled sequentially or through a multiple fragment ligation into a suitable expression vector.

15 The construction of Sections I and II is illustrated in Figures 5 and 6. In the construction of Section I, as shown in Figures 5 and 6, 16 oligonucleotides were assembled into 8 duplexes. The 8 duplexes were then ligated to form Section I. It may  
20 also be noted in Figure 6 that Section I includes an upstream XbaI sticky end and a downstream HindIII sticky end useful for ligation to amplification and expression vectors and for ligation to Section II.

Section II was constructed as shown in  
25 Figures 5 and 6. For this construction, 16 oligonucleotides were assembled into 8 duplexes. The 8 duplexes were then ligated to form Section II as depicted in Figure 6. As also shown in Figure 6, Section II includes an upstream HindIII sticky end and a  
30 downstream EcoRI sticky end useful for ligating into amplification and expression vectors, and to Section I.

Although any suitable vector may be employed to express this DNA, the expression plasmid pCFM1156 may readily be constructed from a plasmid pCFM836, the  
35 construction of which is described in published European Patent Application No. 136,490, and U.S. Patent 4710473

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both of which are hereby incorporated by reference.  
pCFM836 is first cut with Nde I and then blunt-ended  
with Pol I so that both existing Nde I sites are  
destroyed. Next, the vector is digested with Cla I and  
5 Sac II to remove an existing polylinker before ligation  
to a substitute polylinker. This substitute polylinker  
may be constructed according to the procedure of Alton,  
et al., supra. Control of expression in the pCFM1156  
plasmid is by means of a lambda P<sub>L</sub> promoter, which  
10 itself may be under the control of a CI857 repressor  
gene (such as is provided in E. coli strain K12 Htrp or  
FM5). FM5 is described in Burnette et al.  
BIO/TECHNOLOGY Vol. 6, p699 (1988).

Section I was initially cloned into M13 from  
15 XbaI to HindIII and sequenced by the dideoxy method of  
Sanger et al., Proc. Natl. Acad. Sci. USA, 74:5463  
(1977). Section II was cloned into M13 from HindIII to  
EcoR1 and was also sequenced by the dideoxy method.  
Section I was cut out of M13 from XbaI to HindIII and  
20 Section II was cut out of M13 from HindIII to EcoR1.  
These two fragments were then ligated with pCFM1156 cut  
from XbaI to EcoR1 and transformed into E. coli  
strain FM5 (or other suitable E. coli host cells known  
to those skilled in the art) to generate pCFM1156bG-CSF.  
25 This plasmid contains the  $\lambda$ P<sub>L</sub> promoter/operator  
region and has a temperature sensitive replicon. When  
E. coli strain, FM5, harboring pCFM1156bG-CSF is  
cultured at 28°C, the plasmid copy number is maintained  
at 10-20 copies/cell, and transcription from the  $\lambda$ P<sub>L</sub>  
30 promoter is regulated by a temperature sensitive  
repressor. Growth at 42°C results in an increased copy  
number and release of repression at the  $\lambda$ P<sub>L</sub> promoter.  
Recombinant bovine G-CSF begins to accumulate at  
elevated temperatures as the result of promoter  
35 activation and plasmid amplification. The  $\lambda$ P<sub>L</sub> promoter  
lies just upstream from the ribosome binding site and

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the methionine initiation codon of bovine G-CSF. The transcription terminator, t-oop, lies just downstream from the two translational stop codons near the 3' end of the gene. Strains harboring the plasmid, pCFM1156bG-CSF, express r-metbG-CSF at up to 5% of the total cellular protein.

To increase the expression of bovine G-CSF, the synthetic gene was cloned out of pCFM1156bG-CSF into pCFM536 from XbaI to KpnI to generate pCFM 536 bG-CSF1. This construction was transformed into FM5 and bovine G-CSF was expressed at up to 10% of the total protein. Using the oligonucleotide

TTA ATA ATG ATC CCA TTA GGT CCT GCA CGT TCT, site directed mutagenesis (see Example 3 for method) was performed to change the codons of the proline, leucine, and glycine at position 3, 4 and 5 from CCG, CTC, GGC to CCA, TTA and GGT. This new construction, pCFM 536 bG-CSF2, was transformed into FM5, and with this construction bovine G-CSF is expressed at up to 30% of the total protein.

#### E. coli Bovine G-CSF Purification

1. Cell Breakage and Sarkosyl Solubilization and Oxidation

About 100 grams of cell paste was weighed out and resuspended in 2.5 liters water. Cells were dispersed with a suitable mixer until completely dispersed. The suspension through the Gaulin Homogenizer four times at 8000 psig, keeping the temperature below 18°C. The homogenate was centrifuged in a Beckman J2-21 centrifuge using the JA-10 rotor at 10,000 rpm at 4° for thirty minutes.

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The supernatant was decanted and discarded. The pellet was resuspended in 2.5 liters cold water and centrifuged in a J2-21 centrifuge using the JA-10 rotor at 10,000 rpm at 4° for thirty minutes. The supernatant was decanted  
5 and discarded. The pellets were resuspended in 380 ml of water, and 20 ml of 1M Tris pH 8, 100 ml 10% Sarkosyl, and 0.5ml 1% copper sulfate pentahydrate were added. The mixture was stirred overnight at room temperature. The material was centrifuged in the J2-21 centrifuge using  
10 the JA-10 rotor for 30 minutes at 10,000 rpm at 4°. The supernatant was decanted and saved. The pellets were discarded.

## 2. Dowex Removal of Sarkosyl

15

About 500 ml of cold water and 1 liter cold 20 mM Tris pH 8 plus 400 grams Dowex (equilibrated in 20 mM Tris, pH 8) were added to the supernatant. The mixture was stirred at 4° for 90 minutes. The slurry was filtered through a  
20 column and the flow through collected. The resin was washed with 400 ml 20 mM Tris pH 7.7 and this wash was added to the flow through yielding 2.4 liters.

## 3. CM-Sepharose Fast Flow Chromatography

25

The pH of the material was adjusted to 5.4 using 50% acetic acid, and then centrifuged in the J2-21 centrifuge using the JA-10 rotor at 10,000 rpm at 4° for 25  
minutes. The supernatant was decanted and saved. The  
30 pellet was discarded. The supernatant was loaded directly onto a 80 ml CM-Sepharose ion exchange column equilibrated in 20 mM sodium acetate pH 5.4. The column was washed with 100 mM NaCl in starting buffer and then eluted with 150 mM NaCl in starting buffer.  
35 Approximately 850 ml was collected of eluant containing bovine G-CSF.

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#### 4. Diafiltration

The 150 mM NaCl elution from the CM-Sepharose column material was adjusted to pH 3.5 with 0.1N HCl and then  
5 diafiltered using a tangential flow ultrafiltration device (Pellicon) equipped with 10,000 MW membranes against 0.35 mM HCl-Water, pH 3.5. The final volume was adjusted to yield bovine G-CSF at 2 mg/ml.

10

#### EXAMPLE 3

##### Construction of Bovine G-CSF Analogs

This example relates to the use of recombinant methods to general analogs of bovine G-CSF wherein the cysteine at  
15 position 17 was individually replaced by a serine.

Site directed mutagenesis procedures according to Souza, et al., published PCT Application No. WO85/00817, published February 28, 1985, hereby incorporated by reference, were carried out using the  
20 oligonucleotide CTGCTGAAATCCCTCGAACAG.

#### E. coli Bovine G-CSF Analog Purification

##### 1. Cell Breakage and Urea Solubilization and Oxidation 25

About 100 grams of cell paste was weighed out and resuspended in 2.5 liters water. The cells were dispersed with a suitable mixer until completely dispersed. The suspension was passed through a Gaulin  
30 Homogenizer four times at 8000 psig keeping the temperature below 18°C. The homogenate was centrifuged in the J2-21 centrifuge using the JA-10 rotor at 10,000 rpm at 4° for thirty minutes. The supernatant was decanted and discarded. The pellets were resuspended in  
35 2.5 liters cold water and centrifuged in the J2-21 centrifuge using the JA-10 rotor at 10,000 rpm at 4° for

- 37 -

thirty minutes. The supernatant was decanted and discarded. The pellets were resuspended in 120 ml water and 40 ml 1M Tris pH 8.5 and 640 ml 10M Urea were added. The mixture was stirred 2-3 hours at room temperature, and then diluted 1:10 with 20 mM Tris pH 8.5. The mixture was then stirred overnight at room temperature. The material was centrifuged in the Beckman J6-B centrifuge with a JS 4.2 rotor at 4200 rpm at 4° for 45 minutes. The supernatant was decanted and saved. The pellets were discarded. The supernatant was adjusted to pH 5.4 with 50% acetic acid and the material was centrifuged in the J6B using the JS 4.2 rotor at 4200 rpm 4° for 45 minutes. The supernatant was decanted and saved. The mixture was then loaded directly onto a 100 ml CM-Sepharose ion exchange column, equilibrated in 20 mM sodium acetate pH 5.4. The column was washed with 100 mM NaCl in starting buffer then eluted with 150 mM NaCl in starting buffer. Approximately 775 ml of eluant containing bovine G-CSF was collected.

20

## 2. Diafiltration

The 150 mM NaCl elution from the CM-Sepharose column material was adjusted to pH 3.5 with 0.1N HCl and then diafiltered using a tangential flow ultrafiltration device (Pellicon) equipped with 10,000 MW membranes against 0.35 mM HCl-Water, pH 3.5. The final volume was adjusted to yield bovine G-CSF at 2 mg/ml.

30

## EXAMPLE 4

### Method of Treatment of Infection by *Pasteurella Hemolytica*

A G-CSF study was run in cattle challenged with *Pasteurella hemolytica*. Calves weighing approximately 60 Kg were split into two groups of



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eleven. Both groups received 5 ml of bacteria  
( $3 \times 10^7$  /ml) intratracheally on day 1. Four hours  
prior to challenge, the calves were treated with a weak  
solution (pH  $\approx$  5.5) of acetic acid via transtracheal  
5 administration. The group receiving  
subcutaneously-administered human G-CSF (10  $\mu$ g/Kg) had  
been pretreated for 4 days prior to infection and were  
treated daily for the remaining 9 days in the study.

All cattle, except for one in the control  
10 group, showed clinical (subjective scoring) and  
histological signs of pneumonia. Although the G-CSF  
treated cattle were not protected against the infection,  
their clinical status at the end of the study was better  
than the control group, based not only on their clinical  
15 score but also on pathological scoring (%) of lung  
involvement. In addition, 3 cows in the control group  
died while none in the treated group died. Figure 8  
shows white blood cell count (WBC) as a function of  
time. Figure 9 shows neutrophil count as a function of  
20 time. Figure 10 illustrates band count as a function of  
time. Neutrophil count comprises the total of Segs (most  
mature neutrophils) and Bands (neutrophils slightly less  
mature than Segs). Figure 11 shows morbidity as a  
function of time, and Figure 12 shows percent lung  
25 involvement (percent by lung affected) versus time.

#### EXAMPLE 5

##### The Use of G-CSF as a Preventive/Therapeutic Agent for the Control of Bovine Respiratory Disease

30

The purpose of this study was to determine the  
efficacy of human G-CSF in controlling bovine  
respiratory disease (BRD) caused by Pasteurella  
haemolytica. The groups were as follows (10  
35 calves/group):

- 39 -

- Group 1: Infected, unmedicated  
Group 2: Infected, medicated  
(starting 5 days prior challenge)  
Group 3: Infected, medicated (starting  
12 hours prior to challenge)

5 Holstein bull calves at 1-2 days of age were purchased. The calves were placed in individual calf hutches and started on a diet of 1 gallon of milk divided twice per day. At 7 days of age free choice  
10 calf grain was offered, and at 7 days of age free choice water was offered.

Five days prior to challenge (day -5) all calves in group 2 received 10 µg/kg/calf of G-CSF administered SQ, SID. Twelve hours prior to challenge  
15 (day -½) calves in group 3 received 10 µg/kg/calf of G-CSF administered SQ, SID. Calves in group 1 received vehicle control starting at the time of challenge (day 0). Calves remained on medication until day 9 (day before termination of the study).

20 The medication was provided as 0.27 mg/ml solution and administered by injecting 10 µg/kg, SQ, SID. The vehicle control was administered by injecting 2 mls, SQ, SID.

Treatment levels were as follows:

25 Group 1: Vehicle control, Group 2: G-CSF 10 µg/kg, SQ, SID starting on day -5, Group 3: 10 µg/kg, SQ, SID.

The calves were challenged at 25 days of age (day 0). Four hours prior to challenge the calves were treated with a weak solution (pH ≈ 5.5) of acetic acid  
30 via transtracheal administration. Challenge with Pasturella hemolytica was by injecting 5 mls of a  $1 \times 10^8$  overnight broth culture of the challenge organism. Calves were monitored for a total of 10 days.

On day 10 the calves were euthanized and  
35 necropsied. The lungs were scored on the degree of involvement of pneumonia and cultured for bacteriology.

- 40 -

Figure 14 (see Figure 13) shows the relative morbidity of the three groups. Morbidity was based upon body temperature, feed consumption and attitude. Figures 15, 16 and 17 illustrate WBC (white blood count), PMN (neutrophils) and bands versus time, respectively.

#### EXAMPLE 6

##### The Effects of G-CSF on the Incidence and Duration of Respiratory Disease and Immunological and Clinicalpathological Parameters in Calves

The purpose of this study was to determine the effect administering human G-CSF has on the incidence and duration of respiratory disease in calves. Parameters monitored included morbidity, mortality, complete blood counts and response to antigens and recall antigens. The study was conducted in a commercial calf barn.

Sixty (60) holstein bull calves, approximately 21 days of age, were purchased and placed in a calf barn over a 3 day period (day -3 through day -1). As the calves were placed in the barn they were assigned to one of two groups. The calves were placed in individual calf hutches in which there were 30 hutches in a row, with two rows and a 4 foot walkway between the rows. Calves were placed in an alternating manner. An even number of calves were placed each day. Calves were started on the study the day they were placed in the barn. Day 1 of the study occurred when the calf actually was placed in the barn. In other words, there were 3 day 1's, depending on the actual start day. All subsequent days were based on the actual start date.

Starting on day 1, calves in group 1 received daily injections of 10  $\mu$ g/kg of body weight of G-CSF. Calves in group 2 received daily injections of a vehicle control. The injections continued for 21 consecutive days.

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Starting on day 1 the calves were monitored daily for signs of respiratory disease by the animal health technician. The physical signs monitored were body temperature, appetite, respiratory rate and attitude. The monitoring continued for 30 consecutive days (day 1 through day 30). Any calf showing signs of respiratory disease (see diagnosis criteria below) was placed on therapy according to the treatment schedule noted below. All treatments were recorded. Any calf dying during the study was necropsied.

On days 1 (prior to start of injections), 7, 14, 21 and 28 all calves had blood taken for complete blood counts (CBC). In addition blood for CBC's was taken on the 1st and 4th day of respiratory disease. Tests run included packed cell volume (PCV), total red and white cell counts and a differential.

All calves becoming ill were started on therapy. The duration and type of therapy was recorded.

Comparisons were made between groups 1 and 2 for effects of G-CSF on complete blood counts, morbidity due to respiratory disease measured by daily observations and mortality.

#### RESPIRATORY DISEASE MONITORING CRITERIA

25		NORMAL	ABNORMAL
	BODY TEMPERATURE	<103.5	>103.5
	APPETITE	1	2, 3 & 4
30	1=Drink entire amount of milk		
	2=Drink 3/4 of milk		
	3=Drink $\frac{1}{2}$ of milk		
	4=Drink $<\frac{1}{2}$ of milk		

35

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RESPIRATORY RATE

1

2 & 3

1=Normal

2=Slightly elevated

3=Extremely elevated

5

ATTITUDE

1

2, 3 & 4

1=Normal

2=Slightly depressed

3=Moderately depressed

10 4=Severely depressed

### Treatment Schedule

15 Treatment #1: 5 mg/lb oxytetracycline, IM. The duration of therapy was based on the response of the calf. Therapy continued for 2 days after parameters return to normal with a minimum of 4 days and a maximum of 8 days. If calves did not respond to treatment #1, they were started on treatment #2.

20 Treatment #2: 25mg/lb Sulfadimethoxine, PO, 1st day,  
12.5 mg/lb 2nd and subsequent days. Maximum of 5 days.

Figure 18 illustrates the treatment days, for group 1 calves (receiving G-CSF), broken down by first day of treatment (T1), second day of treatment (T2), ...first day of the second course of treatment (T1B), ...fifth day of the second course of treatment (T5B). The treated group consistently has fewer number of total treatments for each treatment point. There is a slight trend to a greater difference between treated and controls as the number of treatments increases. For example, starting at T4, the difference between the treated and control calves increase to about a 40% improvement, compared to 15% at the earlier treatments.

Figure 19 illustrates the total number of  
35 treatments per day for the controls vs. the treated  
calves. Again this shows the reduction in treatments

- 43 -

required for the treated calves vs. the controls. The improvement ranges from close to 50% at times, to 0%. The overall average % improvement for the treated calves is 36%.

5           Figure 20 illustrates the frequency of number of calves, by day, with temperatures above 103.5°F. The data corresponds very well with the total treatment days. There is a consistent improvement in temperatures for the treated calves vs. the controls. The percent  
10 improvement is basically the same as the total treatment days (39.9%).

G-CSF when administered to young veal calves prior to becoming infected with BRD and during infection significantly reduces the severity and length of the  
15 respiratory disease compared to placebo treated controls. G-CSF treated calves had less respiratory disease (27%) compared to controls, and they also became healthier sooner (47% improvement).

20

#### EXAMPLE 7 Klebsiella Pneumonia

The objective of this study was to determine if increasing the circulating neutrophil pool of cells  
25 would allow the dairy cow to respond in a more timely fashion upon bacterial challenge of the mammary gland.

##### G-CSF PROTOCOL:

- 1) The administration of the human G-CSF began two days after arrival and initial evaluation (PM milking)  
30 and continued to be given at the PM milking for a total of 15 days.
- 2) Dose/Route: 10.0 µg/kg daily via subcutaneous administration.
- 3) Control challenge group received 1.0 µg/kg G-CSF  
35 (daily) via subcutaneous administration for 8 days beginning on day 12.

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MASTITIS CHALLENGE MODEL:

- 1) Organism: Klebsiella pneumoniae was given on the morning of the sixth day after the commencement of G-CSF administration.
- 5 2) Dose: 1.0 ml (approximately 200,000 CFU) administered aseptically via the streak canal into the left rear (D) quarter. The remainder of the quarters were used as controls.
- 3) No milk samples were taken for 3 hours after the  
10 challenge in order to allow for the bacteria to have time to establish in the quarter.
- 4) No antibiotic therapeutic intervention was used during the experiment with the exception of fluid therapy if required.

15

EXPERIMENTAL GROUPS:

- |                   |                                   |
|-------------------|-----------------------------------|
| Tx challenge      | (n=3) cow ear tag #: 736,924,964  |
| Tx control        | (n=3) cow ear tag #: 248,293,790  |
| control challenge | (n=3) cow ear tag #: 124,771,4179 |
- 20 Representative results are shown in Figures 21-23.
- Twenty-four hours after the challenge, an independent clinician palpitated all 36 udders. The clinician picked out the infected quarters of the 36 total quarters; these were the challenged quarters
- 25 from the three untreated cows.

**EXAMPLE 8****Method for Prevention of Coliform Mastitis**

- 30 This example is directed to a method using G-CSF in the prevention of coliform mastitis in lactating cows. To test the efficacy of human G-CSF in preventing the clinical effects of coliform mastitis in lactating dairy cows, the cows were given an experimental challenge of a
- 35 pathogenic E. coli.

- 45 -

Twelve mid-lactation Holstein dairy cows were randomly assigned to 1 of 2 groups. Only cows with milk cultures that were negative to coliforms, and with a negative CMT (California Mastitis Test) were used in the experimental study. The two groups were: Group 1 (infected, unmedicated controls) and Group 2 (infected, medicated with 3  $\mu$ g/kg of G-CSF).

The basic method involved treating the cows with G-CSF (group 2) from days 1-17, then challenging on day 10 and finally, monitoring mortality and morbidity for days 11-21. Group 1 received placebo injections of saline for days 1-17.

15

20

25

30

35



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The specific schedule was as follows:

DAY	G-CSF	CBC <sup>1</sup>	CMT <sup>2</sup>	SCC <sup>3</sup>	MILK CULTURE	PLACEBO	BODY TEMPERATURE
0		✓	✓	✓	✓		✓
1	✓					✓	
2	✓					✓	
3	✓					✓	
4	✓					✓	
5	✓		✓			✓	✓
6	✓					✓	
7	✓					✓	
8	✓					✓	
9	✓		✓		✓	✓	✓
10	✓	✓	✓	✓	✓	✓	✓
Challenge							
11	✓		✓		✓	✓	✓
12	✓		✓		✓	✓	✓
13	✓		✓			✓	✓
14	✓	✓	✓	✓		✓	✓
15	✓		✓		✓	✓	✓
16	✓		✓			✓	✓
17	✓		✓			✓	✓
18			✓				✓
19			✓		✓		✓
20			✓				✓
21		✓	✓	✓	✓		✓

<sup>1</sup>Complete Blood Counts, <sup>2</sup>California Mastitis Test, <sup>3</sup>Somatic Cell Counts <sup>4</sup>Supplied by H-13

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All cows were observed twice daily (a.m. and p.m.) and abnormal clinical observations (i.e. appetite, signs of inflammation, ambulation, attitude, etc.) were recorded. The cows had their udders examined once daily (a.m.) during the entire study period with abnormal findings recorded. All injections of test and control compositions were administered between 8 a.m. and 10 a.m. The cows were milked twice daily and all milk was discarded. No other medications were administered during the study period.

The challenge consisted of an intramammary infusion of a virulent culture of E. coli in 1 quarter. The challenge was titrated prior to the start of the study to produce moderate morbidity.

The cows were maintained until day 31 (14 days after the last G-CSF dose) and then sold for slaughter. All cows that died during the study were necropsied and disposed of.

Comparisons between the control and treatment groups were made as to the effectiveness of G-CSF in preventing cows from developing coliform mastitis due to intramammary challenge with E. coli. The results are presented graphically in Figures 24 through 26. The results of milk culture scores (1=1-100 E. coli colonies; 2=100-500 E. coli colonies; 3=greater than 500 E. coli colonies) (Figure 24), clinical scores which are a combination of milk scores (0=normal milk, 1=watery clot, 2=more watery/clot, 3=extremely watery/clot), and udder scores (0=normal, 1=slight swelling/hardness, 2=moderate swelling/hardness, 3=severe swelling/hardness) (Figure 25), and California Mastitis Test (CMT) (negative and trace = 0) scores (Figure 26), show that treatment with G-CSF under the above described experimental conditions was very effective in lessening the severity and accelerating the recovery of the lactating dairy cows. Thus, an effective mastitis treating amount was shown to be 3 µg/kg.

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## EXAMPLE 9

Hemogram Changes in Lactating Dairy Cows  
Given Granulocyte Colony Stimulating Factor  
Following Induction  
of Coliform (E. coli) Mastitis.

5           The original purpose of this study was to determine  
if treatment with G-CSF would hasten recovery from an  
experimentally induced mastitis. The inoculations given  
10 in this experiment did not result in significant clinical  
signs and all cows cleared the infection within 24 hrs  
post inoculation. Hence the therapeutic potential of the  
G-CSF could not be evaluated. The final objective of  
this study was to evaluate the hematologic changes  
15 induced by human G-CSF at a dose of 3 µg/kg on bovine  
peripheral blood.

Eight healthy holstein cows in the second half of  
their lactation were infected with an intramammary  
inoculation of an E. coli isolate. A mastitis derived  
20 E. coli isolate grown for 14-16 hrs in tryptocase soy  
broth was used. Four of the cows were given G-CSF, the  
remaining four comprised an infected control group.

An initial intramammary inoculation of 803.7 CFU was  
given into the right rear quarter. This failed to induce  
25 a clinical mastitis. Second and third inoculations of  
44,000 CFU and 842,000 CFU, respectively, were given 2  
days later and 6 hrs apart. The left rear quarter was  
used for the second and third inoculations. Time zero  
was taken from the time of the second inoculation.

30       Four of these 8 cows were randomly selected and  
injected subcutaneously with human G-CSF at approximately  
6 hrs after inoculation. A single injection was given to  
each of these cows following the afternoon milking, for  
the next 4 days. The dose given was 3 µg/kg each time.  
35 The remaining 4 E. coli challenged cows received no  
treatment.

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Baseline hematology was obtained on three consecutive days pretreatment and on days 0 to 12 post E. coli inoculation. Blood samples were collected from the tail vein into EDTA containing test tubes.

5       The hemogram included total white blood cell count (WBC), a differential cell count, total red cell count (RBC), packed cell volume (PCV) and plasma protein (PlPr) determinations. All samples were processed within 3 hrs of collection and differentials were done on Wright  
10   Giemsa stained smears. Red and white cell counts were done using a Coulter counter model F.

None of the cows showed systemic signs of illness during the experiment. Around 6 hrs post inoculation the infected quarter of some of the cows became swollen, red  
15   and firm. Swelling in the quarters of these cows was only slight by 12 hrs.

Hemograms of the 4 cows given the G-CSF were characterized by a moderate leukocytosis within 24 hrs of the first injection. The noted leukocytosis was  
20   predominantly comprised of mature, nontoxic neutrophils. By the second day of treatment, band neutrophils were seen in the peripheral blood. The greatest left shift was  $2.5 \times 10^3 / \mu l$  bands seen in cow #768. The time at which the maximum left shift  
25   occurred corresponded to the time when the segmented neutrophils reached their highest values. The dose intervals were short enough to result in an additive effect of the drug on neutrophil numbers, which did not return to baseline levels between injections. When the  
30   injections ceased, the cell count declined at a rate similar to the rate of increase.

Around day 7, the counts fell to a level that was about 2-3 times the baseline value and they remained at this level for the rest of the treatment period.

35

- 50 -

Maximum total white blood cell counts and segmented neutrophil counts were seen around day 5. The following table shows these cell counts for the treated and untreated groups on day 0 and day 5. The magnitude of the change (%) in cell counts between day 0 and 5 is shown for the treatment group only.

UNTREATED

10 COUNTS ( $\times 10^3 / \mu l$ )

COW#	Day0		Day5	
	Seg	WBC	Seg	WBC
15 326	3.8	11.6	2.5	8.8
801	2.9	8.3	2.4	7.4
574	4.4	11.1	1.9	6.8
825	3.5	10.2	2.8	8.3

20

G-CSF TREATED

COUNTS ( $\times 10^3 / \mu l$ )

25 COW#	Day0		Day5		% Increase	
	Seg	WBC	Seg	WBC	Seg(%)	WBC(%)
768	2.9	11.7	8.9	21.2	300	180
30 767	4.7	10.4	21.5	28.44	500	270
773	4.2	11	22.6	32.3	500	290
324	4.6	10.3	21.8	32.6	500	320

The untreated group had insignificant changes in cell counts between days 0 and 5. The treated group had 3 to 5 fold increases in their neutrophil counts and 2 to 3 fold increases in their WBC counts.

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Some of the cows (326,801,574,825,767,768) had a small decline in their WBC count 6 hrs post inoculation.

Cow number 324, of the treated group, had a high normal WBC and PlPr and a low normal PCV, prior to and throughout the experiment. These lab abnormalities along with a mild mucopurulent nasal discharge suggested a chronic disease process. Interestingly, this cow had the greatest increase in neutrophil count (500%) with the smallest maximum left shift (<400/u1).

There were no alterations in the numbers of the other white blood cells in the treated and untreated cows throughout the trial. Also, no alterations were observed in red cell parameters or plasma protein, in either group.

It is concluded that:

1. Human G-CSF stimulates bovine neutrophil granulopoiesis.

2. Human G-CSF given daily at a dose of 3 µg/kg causes a 3-5 fold increase in segmented neutrophils after 4 days with only a small left shift.

3. A mild experimentally induced mastitis in one quarter does not impair bone marrow stimulation by the G-CSF.

\* \* \*

The following experiments were designed to determine if G-CSF therapy could prevent mortality in animal models of infectious disease.

#### HAMSTER MODEL OF PSEUDOMONAS AERUGINOSA PNEUMONIA

##### Bacteria

The strain of P. aeruginosa, PA02, used in these experiments was isolated from the blood of a human patient with an abdominal infection. PA02 was cultured

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to exponential growth in trypticase soy broth (TSB) (Difco), and aliquots of this culture were then stored frozen at -70°C in 40% glycerol until used. 1.5 ml aliquots of PAO2 were thawed, added to 50 ml of TSB and  
5 incubated 4 hr in shaker flasks at 37°C. Log-phase organisms were harvested by centrifugation at 2000 X g, washed twice with sterile 0.9% saline, and resuspended to the desired concentration as determined spectrophotometrically. All bacterial inocula were  
10 confirmed by quantitative cultures using a spiral plating instrument (Spiral Systems, Cincinnati).

### Animals

Virus antibody free female Golden Syrian  
15 hamsters weighing 80 to 100 grams were obtained from Charles River Canada (Quebec) and quarantined for 5 days. Groups of 5 animals were housed in cages equipped with individual filter bonnets. Animals were allowed unrestricted access to water and a standard  
20 hamster food (Tecklad, Winfield, Iowa) until the evening prior to infection. Animals were then fasted overnight. Human recombinant Granulocyte Colony Stimulating Factor (G-CSF) or vehicle (5% dextrose in sterile water) was administered subcutaneously in a  
25 total volume of 0.1ml. Animals were administered G-CSF or vehicle on a twice daily (bid) schedule at approximately 8 AM and 3 PM. Injections of G-CSF were continued in surviving animals for 3 days following infection. Animals were infected between 12 and 2 PM.

30 For intratracheal inoculation, hamsters were anesthetized intraperitoneally with 75mg/kg Nembutal (50mg/ml; Abbott). Animals were placed on a 30 degree incline board, jaws were opened, and 0.25ml of the bacterial suspension was administered into the trachea  
35 using a curved 22 gauge needle with a blunted tip 2.5 inches long. Following bacterial inoculation the

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animals were gently shaken for 15 seconds to distribute the inoculum. Animals were allowed to recover from the anesthesia on 37°C heating pads. Only animals which recovered from the anesthesia were used in the experiment. Animals were observed regularly. Animals displaying symptoms of extreme morbidity (inability to walk or remain upright) were euthanized by a blinded observer and counted as a death.

10

## EXAMPLE 10

The Use of G-CSF as a Prophylactic/Therapeutic Agent for the Prevention of Bacterial Pneumonia in Hamsters

15

The purpose of this experiment was to determine the efficacy of various doses of G-CSF in preventing a fatal respiratory infection caused by Pseudomonas aeruginosa. The groups were as follows (15 hamsters/group):

20

Group 1: Infected, unmedicated (vehicle only)

Group 2: Infected, medicated with G-CSF

5ug/kg bid

Group 3: Infected, medicated with G-CSF

15ug/kg bid

25

Group 4: Infected, medicated with G-CSF

50ug/kg bid

30

Dosing of animals was begun on the day prior to bacterial challenge. Thus, animals received 3 doses prior to infection, and surviving animals continued to receive G-CSF bid. Approximately  $5 \times 10^7$  colony forming units (CFU) of PAO2 were administered to each hamster. Figure 27 shows the relative mortality in each group with time. All three groups of hamsters which received G-CSF had significantly less mortality than those animals which received vehicle only.

35



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There was a significant treatment effect (p<.001) for all groups by Log Rank Chi Square Analysis. Hamsters pretreated with G-CSF 5 µg/kg bid prior to infection did not have a significantly decreased mortality rate as compared to control animals. However, animals pretreated with 15 µg/kg G-CSF bid did differ significantly from controls (p<.001). Animals receiving 50 µg/kg bid G-CSF also had significantly decreased mortality (p<.001). The 15 µg/kg bid and 50 µg/kg bid doses were not significantly different from each other.

## EXAMPLE 11

15 Effect of G-CSF Dosing Schedule on Mortality Rate of Hamsters With a Pseudomonas aeruginosa Lung Infection

The purpose of this experiment was to determine the amount of time required between commencement of G-CSF therapy and infectious challenge to effect diminished mortality.

Group 1: Infected, unmedicated (vehicle only)

Group 2: Infected, medicated 15 µg/kg bid beginning approximately 30 hours prior to infection. Animals received 3 doses of G-CSF prior to challenge.

Group 3: Infected, medicated 15 µg/kg bid beginning 5 hours prior to challenge.

Animals received approximately  $5 \times 10^7$  CFU of PAO2 intratracheally. Dosing with G-CSF continued on the bid schedule for 3.5 days in surviving animals. Figure 28 shows the mortality with time in all groups of animals. Both medicated groups had significantly reduced mortality compared to nonmedicated animals.

There was a significant treatment effect (p<.001) for all groups by Log Rank Chi Square Analysis. Hamsters pretreated 5 hours before infection

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had a significantly decreased mortality ( $p < .001$ ) as compared to control animals. Hamsters pretreated 30 hours before infection were also significantly different ( $p < .001$ ) from control animals.

5

\* \* \*

### Rat Model of Candida Albicans Pyelonephritis

#### 10 Organism

The clinical isolate of Candida albicans used in this experiment was maintained on Sabouraud dextrose agar slants. C. albicans was cultured overnight in Sabouraud dextrose broth. C. albicans was isolated by  
15 centrifugation at 2000 X g for 10 min at 4°C. The pellet was washed twice with phosphate buffered saline (PBS) resuspended in PBS, and adjusted to the desired concentration after enumeration using a hemocytometer. All inocula were confirmed by quantitative cultures.

20

#### Animals

Female Sprague-Dawley rats weighing 150-200 grams, obtained from Harlan-Sprague-Dawley, Inc. (Indianapolis, IN), were quarantined for 5 days. Groups  
25 of 4 animals were housed in hanging wire cages allowed unrestricted access to standard rat food and water. Animals received G-CSF or vehicle (PBS) twice daily beginning the day prior to the infection challenge. Surviving animals continued to receive G-CSF or vehicle  
30 bid for 10 days.

For intravenous inoculation, rats were anesthetized with ether. A 0.1 ml volume C. albicans was injected into the tail vein.

Group 1: Infected, unmedicated (vehicle only)

35 Group 2: Infected, medicated with G-CSF  
50ug/kg bid

- 56 -

Beginning approximately 30 hours prior to infection.

Figure 29 shows the mortality with time in each group of animals. Animals received  $1.3 \times 10^6$  CFU of *C. albicans*. There were no deaths in the group which received G-CSF.

There was a significant treatment effect ( $p < .01$ ) for all groups by Log Rank Chi Square Analysis. G-CSF pretreated animals had a significantly decreased mortality rate from control animals ( $p < .001$ ). Rats treated with G-CSF post infection did not differ significantly from the controls.

#### EXAMPLE 12

15

#### G-CSF Administered to Growing Pigs

Bovine G-CSF was administered to growing pigs (~30kg) as follows:

20

Dosage = 5  $\mu$ g/kg body weight/day

Route: subcutaneous

n = 6 pigs

The neutrophil levels were as shown below:

25

<u>Day</u>	<u>Neutrophils (Avg)</u>
0	3108
2	29922
4	24595
6	42713

30

In a study in 6 normal growing pigs, neutrophil levels increased dramatically within 2 days and reached a 13 fold higher level after 6 days of daily administration.

35

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## EXAMPLE 13

## G-CSF Administered to Normal Neonatal Foals

5           Bovine G-CSF was administered to normal neonatal foals commencing at 1 day of age as follows:

Dosage = 10 and 20  $\mu$ g/kg body weight/day

Route: intramuscular

10   n = 5 foals

Day 0 = day prior to first G-CSF administration

The neutrophil level was as shown below:

15	Foal No.	Dose ( $\mu$ g/kg)	Neutrophil Count					
			Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
20	1	20	4950	17473	28072	35524	30874	38263
	2	10	6708	16195	27807	28560	26960	26535
	3	10	5016	10472	6715	16112	19975	16464
	4	10	6480	6225	4400	5610	10332	15372
	5	10	9156	10406	11692	11304	13588	17300

In a study of 5 neonatal foals given bovine G-CSF daily,  
 25 neutrophil levels increased up to three and one-half fold within 24 hours and up to seven and one-half fold after 5 days, depending on dosage.

\* \* \*

30

While the present invention has been described in terms of preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the appended  
 35 claims cover all such equivalent variations which come within the scope of the invention as claimed.

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## WHAT IS CLAIMED IS:

1. A purified and isolated polypeptide having part or all of the primary structural conformation and  
5 one or more of the biological properties of naturally-occurring bovine granulocyte colony stimulating factor and characterized by being the product of prokaryotic or eukaryotic expression of an exogenous DNA sequence.
2. A polypeptide according to claim 1 wherein  
10 the exogenous DNA sequence is a genomic DNA sequence.
3. A polypeptide according to claim 1 wherein the exogenous DNA sequence is a cDNA sequence.
4. A polypeptide according to claim 1 wherein the exogenous DNA sequence is a manufactured DNA  
15 sequence.
5. A polypeptide according to claim 1 wherein the exogenous DNA sequence is carried on an autonomously replicating DNA plasmid or viral vector.
6. A polypeptide according to claim 1  
20 possessing part or all of the primary structural conformation of bovine granulocyte colony stimulating factor as set forth in Figure 2b or any naturally occurring allelic variant thereof.
7. A polypeptide according to claim 1 which  
25 has the immunological properties of naturally-occurring bovine granulocyte colony stimulating factor.
8. A polypeptide according to claim 1 which has the in vitro biological activity of naturally-occurring bovine granulocyte colony stimulating factor.
- 30 9. A DNA sequence for use in securing expression in a prokaryotic or eukaryotic host cell of a polypeptide product having at least a part of the primary structural conformation and one or more of the biological properties of naturally-occurring bovine  
35 granulocyte colony stimulating factor, said DNA sequence selected from among:

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(a) the DNA sequence set out in Figures 2a or its complimentary strands;

(b) DNA sequences which hybridize to the DNA sequences defined in (a) or fragments thereof; and

5 (c) DNA sequences which, but for the degeneracy of the genetic code, would hybridize to the DNA sequences defined in (a) and (b).

10 10. A prokaryotic or eukaryotic host cell transformed or transfected with a DNA sequence according to claim 9 in a manner allowing the host cell to express the polypeptide product.

11. A polypeptide product of the expression of a DNA sequence according to claim 9 in a prokaryotic or eukaryotic host cell.

15 12. A polypeptide product according to claim 11 wherein the polypeptide product is glycosylated or unglycosylated.

20 13. A purified and isolated DNA sequence coding for prokaryotic or eukaryotic host cell expression of a polypeptide having part or all of the primary structural conformation and one or more of the biological properties of bovine granulocyte colony stimulating factor.

25 14. A genomic DNA sequence according to claim 13.

15. A cDNA sequence according to claim 13.

16. A DNA sequence according to claim 16 and including one or more codons preferred for expression in E. coli cells.

30 17. A DNA sequence according to claim 16 and including one or more codons preferred for expression in yeast cells.

35 19. A DNA sequence coding for a polypeptide fragment or polypeptide analog of naturally-occurring bovine granulocyte colony stimulating factor.

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20. A biologically functional plasmid or viral DNA vector containing a DNA sequence according to claim 9.

21. A prokaryotic or eukaryotic host cell stably transformed or transfected with the biologically functional plasmid or viral DNA vector according to claim 20.

22. A biologically functional plasmid or viral DNA vector containing a DNA sequence according to claim 13.

23. A prokaryotic or eukaryotic host cell stably transformed or transfected with the biologically functional plasmid or viral DNA vector according to claim 22.

24. A biologically functional plasmid or viral DNA vector containing a DNA sequence according to claim 19.

25. A prokaryotic or eukaryotic host cell stably transformed or transfected with the biologically functional plasmid or viral DNA vector according to claim 24.

26. A polypeptide product of the expression in a prokaryotic or eukaryotic host cell of a DNA sequence according to claims 13 or 19.

27. A synthetic polypeptide having part or all of the amino acid sequence as set forth in Figure 2b and having one or more of the in vitro biological activities of naturally-occurring bovine granulocyte colony stimulating factor.

28. A synthetic polypeptide having part or all of the secondary conformation of part or all of the amino acid sequence set forth in Figure 2b and having a biological property of naturally-occurring bovine granulocyte colony stimulating factor.

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29. A process for the production of a polypeptide having part or all of the primary structural conformation and one or more of the biological properties of naturally-occurring bovine granulocyte colony stimulating factor, the process comprising: growing, under suitable nutrient conditions, prokaryotic or eukaryotic host cells transformed or transfected with a biologically functional plasmid or viral DNA vector according to claims 20, 22, or 24, and isolating desired polypeptide products of the expression of DNA sequences in the biologically functional plasmid or viral DNA vector.

30. A pharmaceutical composition comprising an effective amount of a polypeptide according to claim 1 and a pharmaceutically acceptable diluent, adjuvant or carrier.

31. A method for treating or preventing mastitis in an animal comprising administering a composition which comprises an effective mastitis treating or preventing amount of granulocyte colony stimulating factor.

32. A method according to claim 31 wherein said administering step comprises administering said granulocyte colony stimulating factor and one or more compounds selected from the group consisting of: GM-CSF, M-CSF, IL3, interferon, IL-2 and TNF.

33. A method according to claim 31 wherein the composition is administered by the parenteral route.

34. A method according to claim 31 wherein the animal is a lactating cow.

35. A method according to claim 31 wherein the animal is a dry cow.

36. A method according to claim 31 wherein the composition is a human granulocyte colony stimulating factor.



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37. A method according to claim 36 wherein the human granulocyte colony stimulating factor is naturally-derived or is derived from genetically engineered host cells containing a genomic DNA sequence, a cDNA sequence or a manufactured DNA sequence encoding human granulocyte colony stimulating factor.

38. A method according to claim 31 wherein the composition is a bovine granulocyte colony stimulating factor.

39. A method according to claim 38 wherein the bovine granulocyte colony stimulating factor is naturally-derived or is derived from genetically engineered host cells containing a genomic DNA sequence, a cDNA sequence or a manufactured DNA sequence encoding bovine granulocyte colony stimulating factor.

40. A method according to claim 38 wherein the bovine granulocyte colony stimulating factor is the polypeptide product of the expression in a prokaryotic or eukaryotic host cell of DNA sequence according to claims 9, 13, or 19.

41. A method for treating or preventing infections in bovine animals comprising administering a composition which comprises a therapeutically effective treating or preventing amount of granulocyte colony stimulating factor.

42. A method as in claim 41 wherein the infection is a bacterial infection.

43. A method as in claim 42 wherein the infection is mastitis or shipping fever.

44. A pharmaceutical composition for treating mastitis in animals comprising a therapeutically effective amount of a human granulocyte colony stimulating factor, in a pharmaceutically acceptable carrier.

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- 63 -

45. A pharmaceutical composition for treating mastitis in animals comprising an effective amount of a bovine granulocyte colony stimulating factor, in a pharmaceutically acceptable carrier.

5           46. A pharmaceutical composition according to claim 45 wherein the bovine granulocyte colony stimulating factor is a polypeptide according to claims 1 or 6.

10           47. A pharmaceutical composition according to claim 45 wherein the bovine granulocyte colony stimulating factor has part or all of the amino acid sequence set forth in Figure 2b and a biological property of naturally-occurring bovine granulocyte colony stimulating factor.

15           48. A pharmaceutical composition according to claim 45 wherein the bovine granulocyte colony stimulating factor is a polypeptide product of the expression in a prokaryotic or eukaryotic host cell of a DNA sequence to Claims 9, 13, or 19.

20           49. A pharmaceutical composition according to Claims 44, 45, 46, 47 or 48 which is parenteral unit dosage form.

25           50. A pharmaceutical composition according to Claims 44, 45, 46, 47 or 48 which is in an aqueous pharmaceutical carrier and suitable for intravenous or subcutaneous administration.

30           51. A pharmaceutical composition according to Claims 44, 45, 46, 47 or 48 which is in lyophilized form and upon rehydration is suitable for intramuscular or subcutaneous administration.

35           52. A prophylactic treatment for the prevention of an infection in a normal animal comprising administering to the normal animal a prophylactically effective amount of granulocyte colony stimulating factor.

- 64 -

53. A method as in Claim 52 wherein the normal animal is a mammal selected from the group consisting of humans, bovine animals, canine animals, feline animals, equine animals and porcine animals.

5 54. A method as in Claim 53 wherein the animal is human and the infection is selected from the group consisting of pneumonia, surgical wound infections, intestinal infections, and puncture wounds.

10 55. A method as in Claim 53 when the animal is bovine and the infection is selected from the group consisting of mastitis and shipping fever.

56. A method as in Claim 53 wherein the animal is equine and the infection is neonatal septicemia.

15 57. A method as in Claim 53 wherein the animal is porcine and the infection is pleuropneumoniae.

58. A process for isolating and purifying G-CSF from a G-CSF producing microorganism comprising:

20 1) lysing the microorganism and separating insoluble material containing G-CSF from soluble proteinaceous material;  
2) solubilizing and oxidizing the G-CSF in the presence of a denaturant solubilizing agent and an oxidizing agent;

25 3) removing the denaturant solubilizing agent from the G-CSF;

4) subjecting the G-CSF to ion exchange chromatography; and

5) recovering purified G-CSF.

30 59. A process according to Claim 58 wherein the G-CSF is met-hG-CSF.

60. A process according to Claim 58 wherein step 4) comprises ion exchange chromatography step is an anion exchange chromatography step followed by cation  
35 exchange chromatography.

- 65 -

61. A process according to Claim 58 wherein the denaturant solubilizing agent of step 2) is Sarkosyl.

62. A process according to Claim 58 wherein  
5 the oxidizing agent is  $\text{CuSO}_4$ .

63. A process according to Claim 58 wherein step 3) the denaturant solubilizing agent is removed using Dowex.

64. A process according to Claim 58 wherein  
10 G-CSF is hG-CSF and after step 1) is the step of extracting the G-CSF material with deoxycholate.

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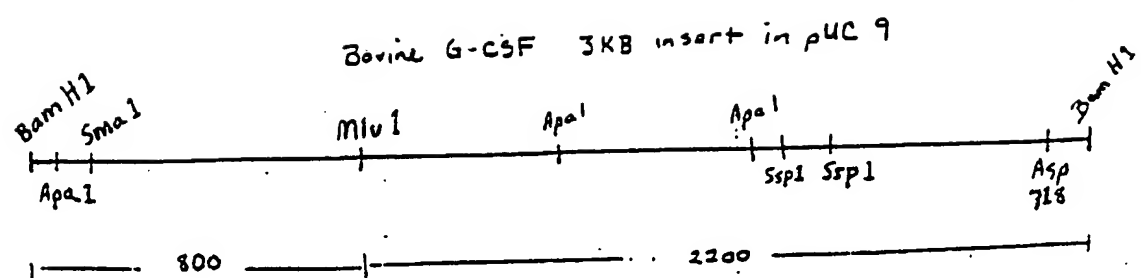


FIGURE 1

RESTRICTION MAP and STRATEGY

Figure 2a

bovine G-CSF sequence  
bqcsf.dna

```

1  ACCCCCCCTTG GCCCTGCCCG ATCCCTGCCC CAGAGCTTCC TGCTCAAGTG
51  CTTAGACCAA GTGAGGAAAA TCCAGGCTGA TGGCGCCGAG CTGCAGGAGA
101 GGCTGTGTGC CGCCCACAAG CTGTGCCACC CGGAGGAGCT GATGCTGCTC
151 AGGCACTCTC TGGGCATCCC CCAGGCTCCC CTAAGCAGCT GCTCCAGCCA
201 GTCCCTGCAG CTGCGAGGCT GCCTGAACCA ACTACACGGC GGCCTCTTTC
251 TCTACCAGGG CCTCCTGCAG GCCCTGGCGG GCATCTCCCC AGAGCTGGCC
301 CCCACCTTGG ACACACTGCA GCTGGACGTC ACTGACTTTG CCACGAACAT
351 CTGGCTGCAG ATGGAGGACC TGGGGGUGGC CCCCGCTGTG CAGCCCACCC
401 AGGGCCCCAT GCGGACCTTC ACTTCAGCCT TCCAACGCAG AGCAGGAGGG
451 GTCTTGCTTG CTTCCCAGCT GCATCGTTTC CTGGAGCTGG CATACCGTGG
501 CCTGCGCTAC CTTGCTGAGC CTTGA

```

Figure 2b

```

1  [Thr Pro Leu Gly Pro Ala Arg Ser Leu Pro Gln Ser Phe Leu Leu
46  Lys Cys Leu Glu Gln Val Arg Lys Ile Gln Ala Asp Gly Ala Glu
91  Leu Gln Glu Arg Leu] [Cys Ala Ala His Lys Leu Cys His Pro Glu
136 Glu Leu Met Leu Leu Arg His Ser Leu Gly Ile Pro Gln Ala Pro
181 Leu Ser Ser Cys Ser Ser Gln Ser Leu Gln Leu] [Arg Gly Cys Leu
226 Asn Gln Leu His Gly Gly Leu Phe Leu Tyr Gln Gly Leu Leu Gln
271 Ala Leu Ala Gly Ile Ser Pro Glu Leu Ala Pro Thr Leu Asp Thr
316 Leu Gln Leu Asp Val Thr Asp Phe Ala Thr Asn Ile Trp Leu Gln]
361 [Met Glu Asp Leu Gly Ala Ala Pro Ala Val Gln Pro Thr Gln Gly
406 Ala Met Pro Thr Phe Thr Ser Ala Phe Gln Arg Arg Ala Gly Gly
451 Val Leu Val Ala Ser Gln Leu His Arg Phe Leu Glu Leu Ala Tyr
496 Arg Gly Leu Arg Tyr Leu Ala Glu Pro End]

```

GGGGACAGGCTTGAGAAATCCAAAGGAGAGGGGCAAGGACACTGCCCGCAAGTCTGCCAGAGCAGAGGGGAGACCCGACTCAGCTGCCACTTCCC 100  
CACAGGCTCGTGGCGCTCCAGGCGTCTATCAGCGGCTCAGCCTTGTTCAGCTGTTCTGTTCAAACACTCTGGGGCCATTACGGCTGGGTGGGGCAGC 200  
GGGAGGAAGGGAGTTTATGGGGGGCAAGGCGACGCTCAAGGAGGATCAGAGATTCCACAATTTCAAAAACCTTTCGAAACAGCTTTTGTTCACACCC 300  
CCTGCATTGTCTTGGACACCAAAATTGCATAAATCTGGGAAGTATTACTAAGCCTTAGTCGTGGCCCCAGGTAATTTCTCCAGGCCCTCCATGGGGT 400

-30  
MetAlaGlyProAlaThrGlnSerProM 500  
TATGTATAAAGGGCCCCCTAGAGCTGGGCCCCAAAACAGCCCGAGCCTGCAGCCAGCCCCACCCAGACCCATGGCTGGACCTGCCACCCAGAGCCCCA

-20 -18  
etLysLeuMetA 600  
TGAAGCTGATGGCTGAGTGTCTTGGCCAGGATGGGAGAGCCGCTGCCCTGGCATGGGAGGGAGGCTGGTGTGACAGAGGGGCTGGGGATCCCCGTCT

-18  
LeuGlnLeuL 700  
GGGAATGGGGATTAAAGGCACCCAGTGTCCCGAGAGGGCTCAGGTGGTAGGGAACAGCATGTCTCTGAGCCCGCTCTGTCCCCAGCTGCAGCTGC

-10 -1 +1 10 20  
euLeuTrpHisSerAlaLeuTrpThrValGlnGluAlaThrProLeuGlyProAlaSerSerLeuProGlnSerPheLeuLeuLysCysLeuGluGlnVa 800  
TGCTGTGGCACAGTGCAGCTCTGGACAGTGCAGGAAGCCACCCCTGGGCCCTGCCAGCTCCCTGCCAGAGCTTCTGTCTCAAGTGTCTAGAGCAAGT

30 35  
IArgLysIleGlnGlyAspGlyAlaAlaLeuGlnGluLysLeu 900  
GAGGAAGATCCAGGGCGATGGCGCAGCGCTCCAGGAGAAGCTGTGTAGTGAAGTGGGTGAGAGGCTGTGGAGGGAAGCCGGTGGGAGAGCTAAGGGG

GA TGGAACTGCAGGGCCAACATCTCTGGAAGGGACATGGGAGAATATTAGGAGCAGTGGAGCTGGGGAAGCTGGGAAGGACTGGGGAGGAGGACCT 1000  
TGGTGGGACAGTGTCTGGGAGGGCTGGCTGGGATGGGAGTGGAGGCATCATTTAGGAGAAAGGGCAAGGGCCCTGTGAGATCAGAGAGTGGGGGTG 1100  
CAGGGCAGAGAGGAAC TGAACAGCCTGGCAGGACATGGAGGGAGGGGAAGACCAGAGAGTGGGGAGGACCCGGGAAGGAGCGGACCCGGCCACGGC 1200

36 40 50  
CysAlaThrTyrLysLeuCysHisProGluGluLeuValLeuLeuGlyHisSerLeuGlyIleProTrpA 1300  
GAGTCTCACTCAGCATCTTCCATCCCCAGTGTGCCACCTACAAGCTGTGCCACCCGAGGAGCTGGTGTCTGCTGGACACTCTCTGGGATCCCTGGG

60 70 71  
IaProLeuSerSerCysProSerGlnAlaLeuGlnLeu 1400  
CTCCCTTGAGCAGCTGCCAGCCAGGCCCTGCAGCTGTGAGTGTGAGGAAAGGATAAGGCTAATGAGGAGGGGAAGGAGAGGAGGAACCCATGGG

72  
AlaGlyCysLeuSerGln 1500  
CTCCCCATGTCTCCAGGTTCGAAGCTGGGGGCTGACGTATCTCAGGCAGCACCCTTAACCTCTCCGCTCTGTCTCACAGGAGGCTGCTTGAAGCAA

80 90 100 110  
LeuHisSerGlyLeuPheLeuTyrGlnGlyLeuLeuGlnAlaLeuGluGlyIleSerProGluLeuGlyProThrLeuAspThrLeuGlnLeuAspValA 1600  
CTCCATAGCGGCTTTCTCTACAGGGGCTCTGACGGCCCTGGGAAGGATCTCCCCGAGTGGGTCCACCTTGACACACAC\*GAGCTGGAGCTGC

120  
IaAspPheAlaThrThrIleTrpGlnGln 1700  
CCGACTTTGCCACCACTCTGGCAGCAGTGTAGCCTTGTGGGAGGGTGGCCAAAGGTCTGTGTCATTCTGGGCACCACAGCCGGGCTGTGTATGG

121  
MetGluG 1800  
GCCCTGTCCATGTCTGCAGCCCCAGCATTTCCTCATTGTATAACGCCCACTCAGAAGGGCCCAACCACTGATCACAGCTTTCCCCCAGATGGAAG

130 140 150  
IuLeuGlyMetAlaProAlaLeuGlnProThrGlnGlyAlaMetProAlaPheAlaSerAlaPheGlnArgArgAlaGlyGlyValLeuValAlaSerHI 1900  
AAGTGGGAATGGCCCTGCCCTGACGCCACCCAGGGTGCATGCGGCTCTGCTTCCAGCGCCGGGAGGAGGGTCTGGTGGCTCCCA

160 170 174  
sLeuGlnSerPheLeuGluValSerTyrArgValLeuArgHisLeuAlaGlnProOP 2000  
TCTGCAGAGCTTCCTGGAGGTGTCTACCGCTTCTACGCCACTTGCCAGCCCTGAGCCAAGCCCTCCCATCCATGTATTATCTCTATTTAATAT

TTATGTCTATTAAAGCCTCATATTAAAGACAGGGAAGACAGAACGGAGCCCCAGGCCCTGTGTCTCTCCATTTCTGAGTTTCATCTCTCTGCC 2100  
TGTAGCAGTGAAGAAAGCTCTGTCTCCATCCCTGGACTGGGAGGTAGATAGGTAATACCAAGTATTATTACTATGACTGTCCCCAGCCCTGG 2200  
CTCTGCAATGGCACTGGGATGAGCCGCTGTGAGCCCTGTGCTGAGGGTCCCCACCTGGGACCCCTTSAGAGTATCAGGTCTCCACGTGGGAGACAAG 2300  
AAATCCCTGTTTAAATATTAAACAGCAGTGTTCCTCATCTGGGTCTTGACCCCTCACTCTGGGCTCAGCCGACTGCACAGCGGCCCTGCATCCCTT 2400  
GGCTGTGAGGCCCTTGACAAAGCAGAGGTGGCCAGAGCTGGGAGGCATGGCCCTGGGGTCCCAAGATTTGCTGGGGAATCTCGTTTTCTTCTTAAGAC 2500  
TTTGGGACATGGTTTGTACTCCBAACATCAGCAGTGTCTCTGTTTTCTGGGTGGCTCGGGACACCTGCCCTGCCCCCAGGAGGTGAGGACTGT 2600  
GACTCTTTTATGGGCCAGGAGGTGCTGGACATTGCTTGTGGATGGGACTGGGATGTGGGAGGAGCAGACAGGGAATCATGTGAGGCTGT 2700  
GTGTAAAGGAAGCTCCACTGTCACTTCCACCTCTTCAACCCCACTCACCAGTGTCCCTCCACTGTACATTGTAAGTGAAGTTCAGGATTAATAAG 2800  
TGTTCCTCTCACTCACGTCTTCTCTCTTGTAGTCCAGCTGGTGGCTGGCCAGGGGCTGGGAGGTGGCTGAAGGTTGGAGAGGCCAGAGGGAGGT 2900  
CGGGAGGAGGCTGGGGAGGAGGTCCAGGGAGGAGGAGAAAGTTCGAAGTCTGTGACATTATTCGTTAGCACATATTATCTGAGCACCTACT 3000  
CTGTGCAGACGCTGGGTAAGTGTGGGGACACAGCAGGGAACAAGGCAGACATGGAATCTGCACTCGAG 3070

Figure 3

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SGCSF.dna

```

                                30                                60
TTC TAG AAA AAA CCA AGG AGG TAA TAA ATA ATG ACT CCG CTG GGT CCG GCA CGT TCT CTG
Phe End Lys Lys Pro Arg Arg End End Ile Met Thr Pro Leu Gly Pro Ala Arg Ser Leu

                                90                                120
CCG CAG TCT TTC CTG CTG AAA TGC CTG GAA CAG GTT CGT AAA ATC CAG GCT GAC GGT GCA
Pro Gln Ser Phe Leu Leu Lys Cys Leu Glu Gln Val Arg Lys Ile Gln Ala Asp Gly Ala

                                150                                180
GAG CTC CAG GAA CGT CTG TGC GCT GCG CAC AAA CTG TGC CAC CCG GAA GAG CTG ATG CTG
Glu Leu Gln Glu Arg Leu Cys Ala Ala His Lys Leu Cys His Pro Glu Glu Leu Met Leu

                                210                                240
CTC CGT CAC TCT CTG GGT ATC CCG CAG GCG CCG CTG TCT TCC TGC TCT TCC CAG TCT CTG
Leu Arg His Ser Leu Gly Ile Pro Gln Ala Pro Leu Ser Ser Cys Ser Ser Gln Ser Leu

                                270                                300
CAG CTC CGT GGT TGC CTG AAC CAG CTC CAT GGT GGC CTG TTC CTG TAC CAG GGT CTG CTC
Gln Leu Arg Gly Cys Leu Asn Gln Leu His Gly Gly Leu Phe Leu Tyr Gln Gly Leu Leu

                                330                                360
CAA GCT TTG GCA GGT ATC TCT CCG GAA CTG GCT CCG ACC CTG GAC ACC CTG CAG CTC GAC
Gln Ala Leu Ala Gly Ile Ser Pro Glu Leu Ala Pro Thr Leu Asp Thr Leu Gln Leu Asp

                                390                                420
GTT ACC GAC TTC GCT ACC AAC ATC TGG CTG CAG ATG GAA GAT CTG GGT GCT GCA CCG GCG
Val Thr Asp Phe Ala Thr Asn Ile Trp Leu Gln Met Glu Asp Leu Gly Ala Ala Pro Ala

                                450                                480
GTT CAG CCG ACC CAG GGC GCT ATG CCG ACC TTC ACC TCT GCT TTC CAG CGT CGC GCT GGT
Val Gln Pro Thr Gln Gly Ala Met Pro Thr Phe Thr Ser Ala Phe Gln Arg Arg Ala Gly

                                510                                540
GGC GTT CTG GTA GCT TCT CAG CTG CAC CGT TTC CTC GAG CTG GCT TAC CGT GGT CTG CGT
Gly Val Leu Val Ala Ser Gln Leu His Arg Phe Leu Glu Leu Ala Tyr Arg Gly Leu Arg

TAC CTG GCT GAA CCG TAA TAG GAT CC
Tyr Leu Ala Glu Pro End End Asp

```

Figure 4



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dGCSF Un24

```

      10      20      30      40      50      60
CTAGAAAA AACCAAGGAG GTAATAAATA ATGACTCCGC TCGGCCCTGC ACGTTCTCTG
      TTTT TTGGTTCCTC CATTATTTAT TACTGAGGCG AGCCGGGGACG TGCAAGAGAC

      70      80      90      100     110     120
CCGCAGAGCT TCCTGCTGAA ATGCCTCGAA CAGGTTCTGA AAATTCAGGC TGATGGTGCT
GGCGTCTCGA AGGACGACTT TACGGAGCTT GTCCAAGCAT TTTAAGTCCG ACTACACGA

      130     140     150     160     170     180
GAGCTCCAGG AGCGTCTGTG CGCTGCTCAC AAGCTCTGCC ACCCTGAAGA ACTCATGCTG
CTCGAGGTCC TCGCAGACAC GCGACGAGTG TTCGAGACGG TGGGACTTCT TGAGTACGAC

      190     200     210     220     230     240
CTCCGTCACT CTCTGGGGAT CCCGAGGCG CGCTGTCTT CTTGCTCCTC TCAGTCTCTG
GAGGCAGTGA GAGTCCCTA GGGCGTCCGC GGCACAGAA GAACGAGGAG AGTCAGAGAC

      250     260     270     280     290     300
CAACTCCGTG GTTGCCTGAA CCAGCTCCAT GGTGGCCTGT TCCTGTACCA GGTCTCTCTG
GTTGAGGCAC CAACGGACTT GGTGAGGTA CCACCGGACA AGGACATGCT CCCAGAGGAC

      310     320     330     340     350     360
CAAGCTTTGG CAGGCATCTC TCCGGAAGTC GCACCTACTC TCGATACTCT GCAGCTCGAC
GTTCGAACC GTCCGTAGAG AGGCCTTGAG CGTGGATGAG AGCTATGAGA CGTCGAGCTG

      370     380     390     400     410     420
GTTACCGACT TCGCTACCAA CATTTGGCTC CAGATGGAAG ATCTGGGCGC TGCACCGGCT
CAATGGCTGA AGCGATGGTT GTAAACCGAG GTCTACCTTC TAGACCCGCG ACGTGGCCCA

      430     440     450     460     470     480
GTTCAGCCGA CTCAGGCGC TATGCCTACC TTCACCTCTG CTTTCAGCG TCGTCTCTGT
CAAGTCGGCT GAGTCCCGCG ATACGGATGG AAGTGGAGAC GAAAGGTGCG AGCAGGACCA

      490     500     510     520     530     540
GGTGTTCCTG TAGCTTCTCA GCTGCACCGT TTCCTCGAGC TGGCTTACCG TGCTCTGCGT
CCACAGAGAC ATCGAAGAGT CAGCTGGCA AAGGAGCTCG ACCGAATGG ACCAGACGCA

      550     560
TACCTGGCTG AACCGTAATA G
ATGGACCGAC TTGGCATTAT CTTAA

```

Figure 5

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bGCSF dna4

Figure 6

```

      12      22      32      42      52      62
CTAGAAAAAA CCAAGGAGGT AATAAATAAT GACTCCGCTC GGCCCTGCAC GTTCTCTGCC
      TTTTTT GGTTCCTCCA TTATTTATTA CTGAGGCGAG CCGGGACGTG CAAGAGACGG

      72      82      92      102     112     122
GCAGAGCTTC CTGCTGAAAT GCCTCGAACA GGTTCGTAAA ATTCAGGCTG ATGGTGCTGA
CGTCTCGAAG GACGACTTTA CGGAGCTTGT CCAAGCATT TAAGTCCGAC TACCACGACT

      132     142     152     162     172     182
GCTCCAGGAG CGTCTGTGCG CTGCTCACAA GCTCTGCCAC CCTGAAGAAC TCATGCTGCT
CGAGGTCCTC GCAGACACGC GACGAGTGTT CGAGACGGTG GGACTTCTTG AGTACGACGA

      192     202     212     222     232     242
CCGTCACTCT CTGGGGATCC CGCAGGCGCC GCTGTCTTCT TGCTCCTCTC AGTCTCTGCA
GGCAGTGAGA GACCCCTAGG GCGTCCGCGG CGACAGAAGA ACGAGGAGAG TCAGAGACGT

      252     262     272     282     292     302
ACTCCGTGGT TGCCTGAACC AGCTCCATGG TGGCCTGTTC CTGTACCAGG GTCTCCTGCA
TGAGGCACCA ACGGACTTGG TCGAGGTACC ACCGGACAAG GACATGGTCC CAGAGGACGT

```

TCGA

bGCSF SUBUNIT I XbaI-HindIII

bGCSF dna4

```

      312     322     332     342     352     362
AGCTTTGGCA GGCATCTCTC CGGAACTCGC ACCTACTCTC GATACTCTGC AGCTCGACGT
      AACCGT CCGTAGAGAG GCCTTGAGCG TGGATGAGAG CTATGAGACG TCGAGCTGCA

      372     382     392     402     412     422
TACCGACTTC GCTACCAACA TTTGGCTCCA GATGGAAGAT CTGGGCGCTG CACCGGCTGT
ATGGCTGAAG CGATGGTTGT AAACCGAGGT CTACCTTCTA GACCCGCGAC GTGGCCGACA

      432     442     452     462     472     482
TCAGCCGACT CAGGGCGCTA TGCCTACCTT CACCTCTGCT TTCCAGCGTC GTGCTGGTGG
AGTCGGCTGA GTCCCGCGAT ACGGATGGAA GTGGAGACGA AAGGTCGCGA CACGACCACC

      492     502     512     522     532     542
TGTTCTGGTA GCTTCTCAGC TGCACCGTTT CCTCGAGCTG GCTTACCGTG GTCTGCGTTA
ACAAGACCAT CGAAGAGTCG ACGTGGCAAA GGAGCTCGAC CGAATGGCAC CAGACGCAAT

      552     562
CCTGGCTGAA CCGTAATAG
GGACCGACTT GGCATTATCT TAA

```

bGCSF SUBUNIT II HindIII-EcoRI

bGCSF dna4

```

          30                                60
C TAG AAA AAA CCA AGG AGG TAA TAA ATA ATG ACT CCG CTC GGC CCT GCA CGT TCT CTG
          Met Thr Pro Leu Gly Pro Ala Arg Ser Leu

          90                                120
CCG CAG AGC TTC CTG CTG AAA TGC CTC GAA CAG GTT CGT AAA ATT CAG GCT GAT GGT GCT
Pro Gln Ser Phe Leu Leu Lys Cys Leu Glu Gln Val Arg Lys Ile Gln Ala Asp Gly Ala

          150                                180
GAG CTC CAG GAG CGT CTG TGC GCT GCT CAC AAG CTC TGC CAC CCT GAA GAA CTC ATG CTG
Glu Leu Gln Glu Arg Leu Cys Ala Ala His Lys Leu Cys His Pro Glu Glu Leu Met Leu

          210                                240
CTC CGT CAC TCT CTG GGG ATC CCG CAG GCG CCG CTG TCT TCT TGC TCC TCT CAG TCT CTG
Leu Arg His Ser Leu Gly Ile Pro Gln Ala Pro Leu Ser Ser Cys Ser Ser Gln Ser Leu

          270                                300
CAA CTC CGT GGT TGC CTG AAC CAG CTC CAT GGT GGC CTG TTC CTG TAC CAG GGT CTC CTG
Gln Leu Arg Gly Cys Leu Asn Gln Leu His Gly Gly Leu Phe Leu Tyr Gln Gly Leu Leu

          330                                360
CAA GCT TTG GCA GGC ATC TCT CCG GAA CTC GCA CCT ACT CTC GAT ACT CTG CAG CTC GAC
Gln Ala Leu Ala Gly Ile Ser Pro Glu Leu Ala Pro Thr Leu Asp Thr Leu Gln Leu Asp

          390                                420
GTT ACC GAC TTC GCT ACC AAC ATT TGG CTC CAG ATG GAA GAT CTG GGC GCT GCA CCG GCT
Val Thr Asp Phe Ala Thr Asn Ile Trp Leu Gln Met Glu Asp Leu Gly Ala Ala Pro Ala

          450                                480
GTT CAG CCG ACT CAG GGC GCT ATG CCT ACC TTC ACC TCT GCT TTC CAG CGT CGT GCT GGT
Val Gln Pro Thr Gln Gly Ala Met Pro Thr Phe Thr Ser Ala Phe Gln Arg Arg Ala Gly

          510                                540
GGT GTT CTG GTA GCT TCT CAG CTG CAC CGT TTC CTC GAG CTG GCT TAC CGT GGT CTG CGT
Gly Val Leu Val Ala Ser Gln Leu His Arg Phe Leu Glu Leu Ala Tyr Arg Gly Leu Arg

TAC CTG GCT GAA CCG TAA TAG AAT TC
Tyr Leu Ala Glu Pro End End

```

Figure 7

FIGURE 8

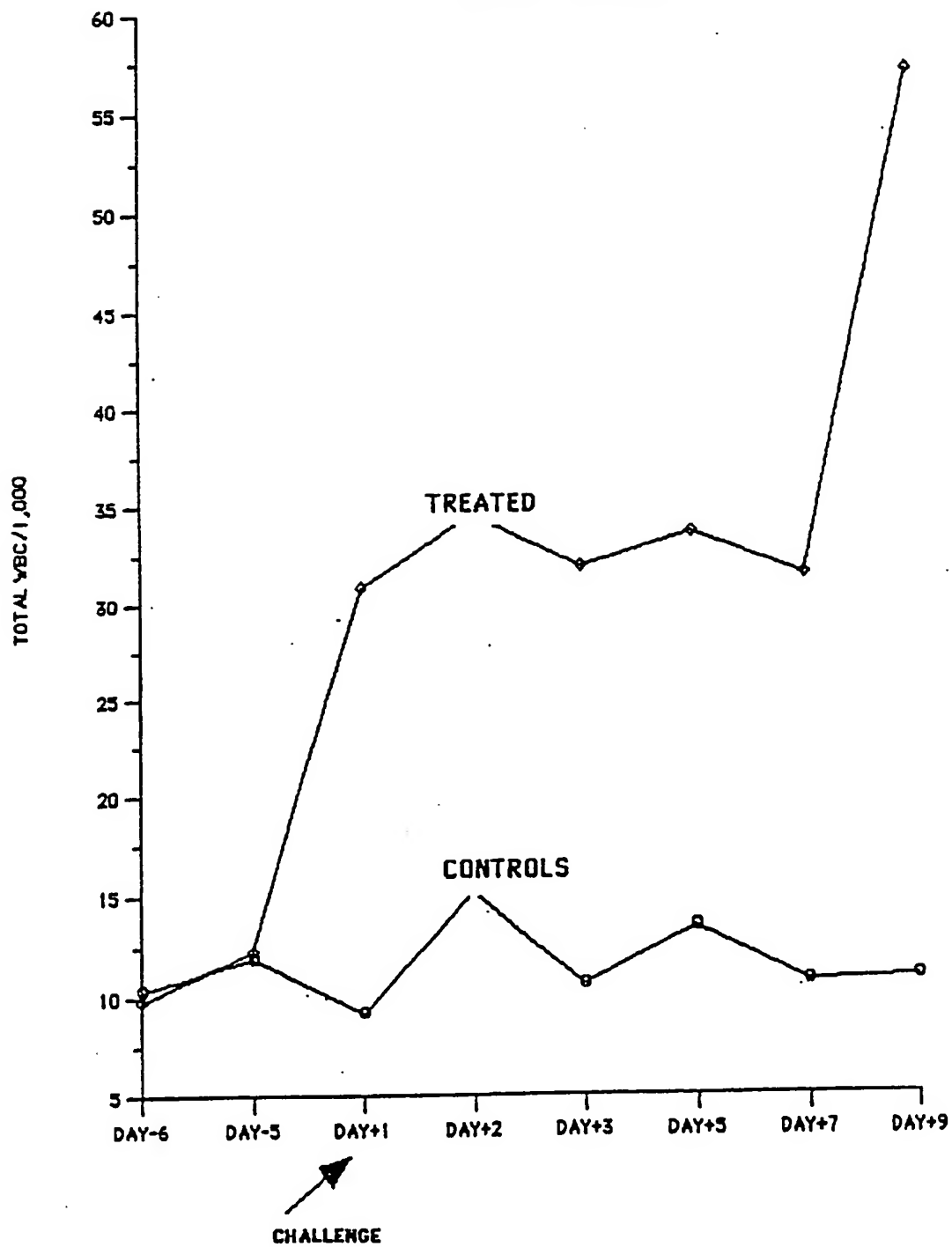
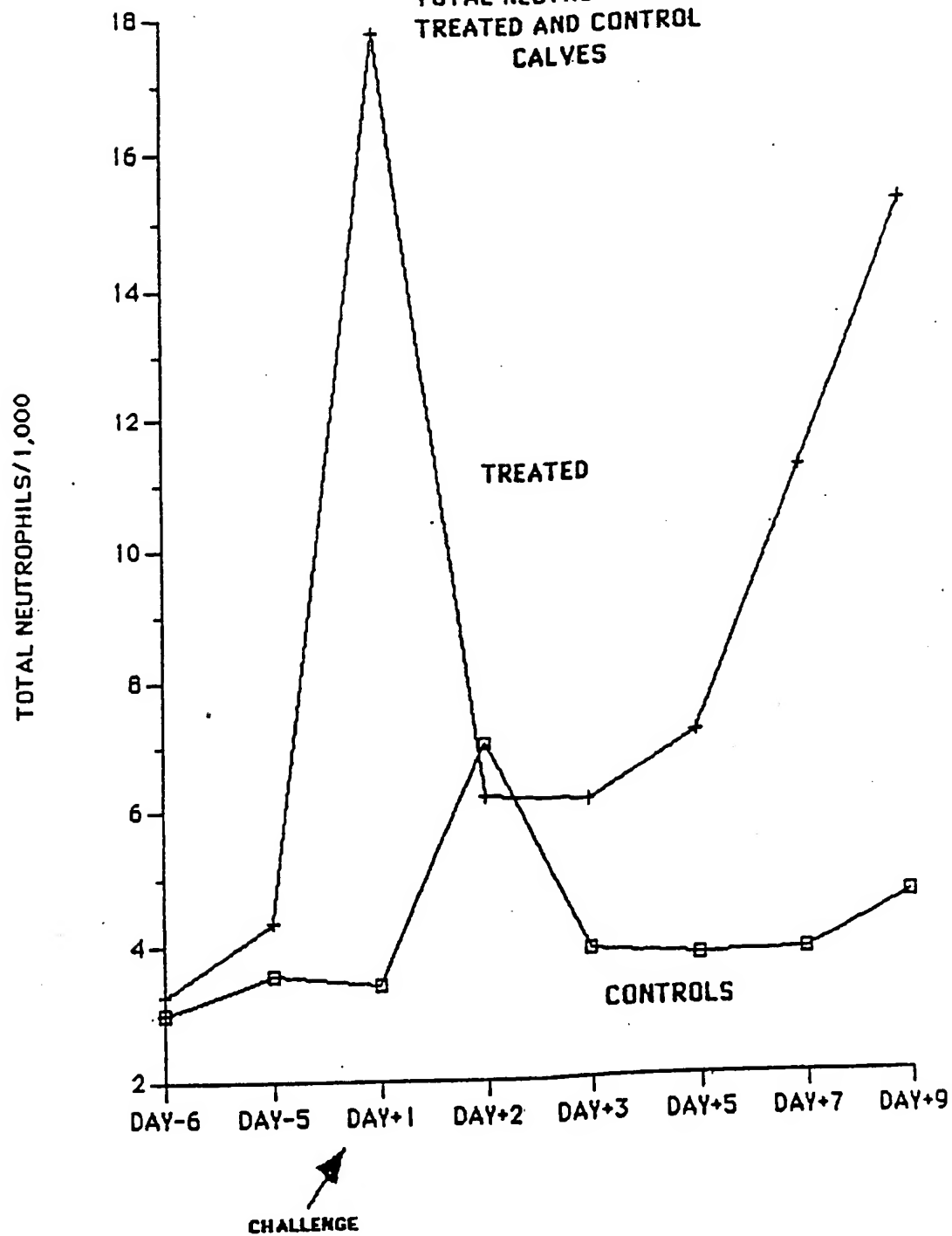
TOTAL WBC IN TREATED AND  
CONTROL CALVES

FIGURE 9

TOTAL NEUTROPHILS IN  
TREATED AND CONTROL  
CALVES

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FIGURE 10

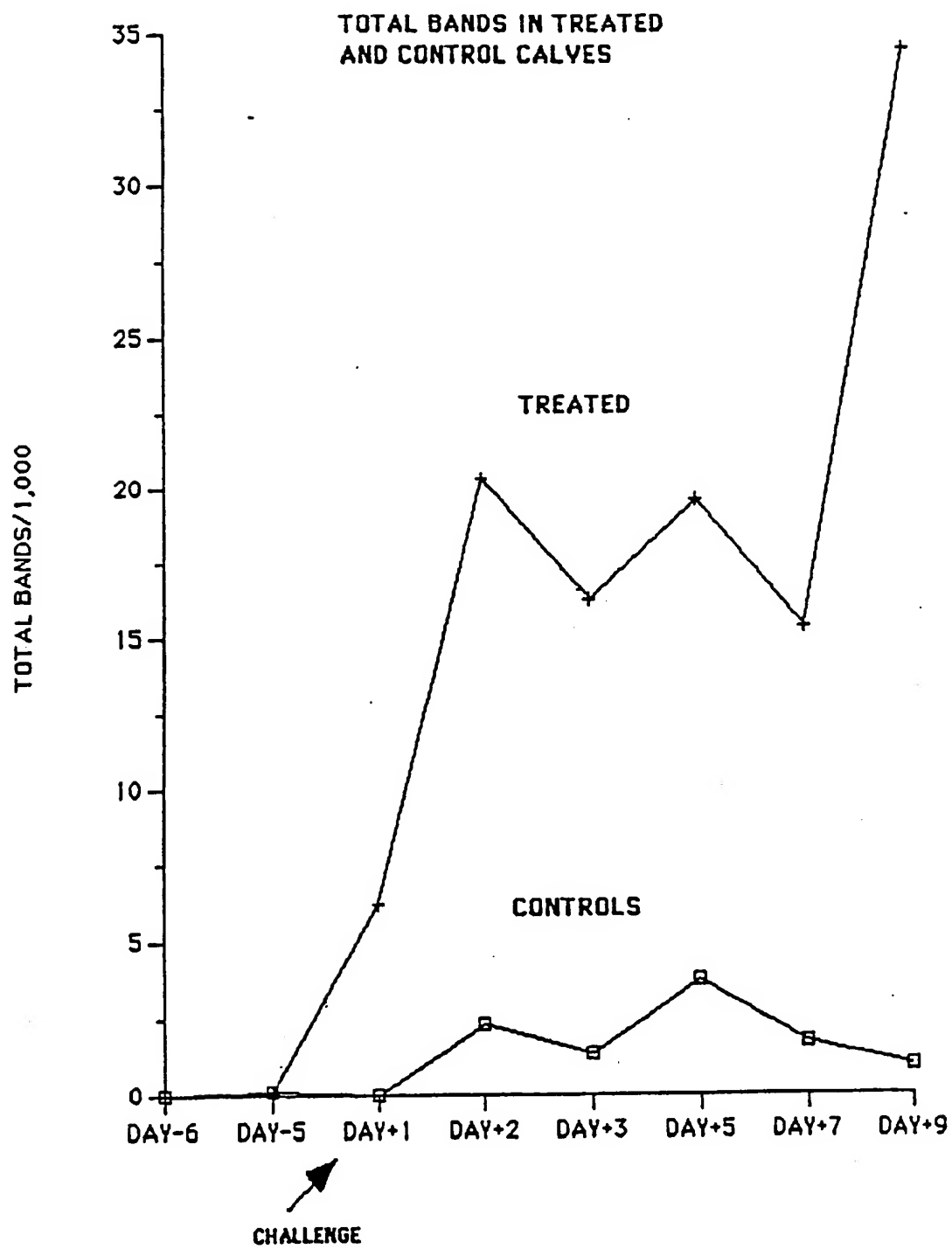


FIGURE 11

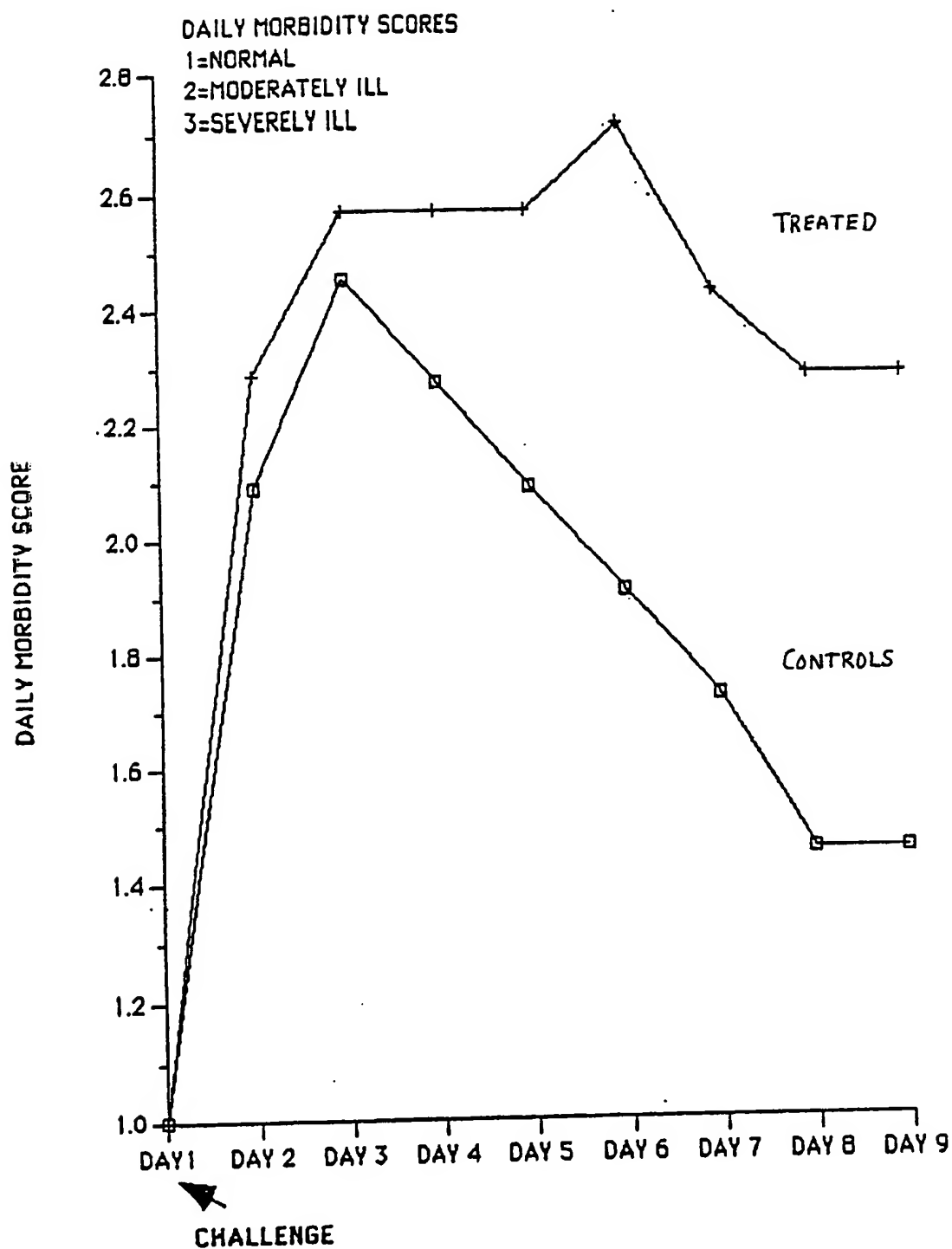


FIGURE 12

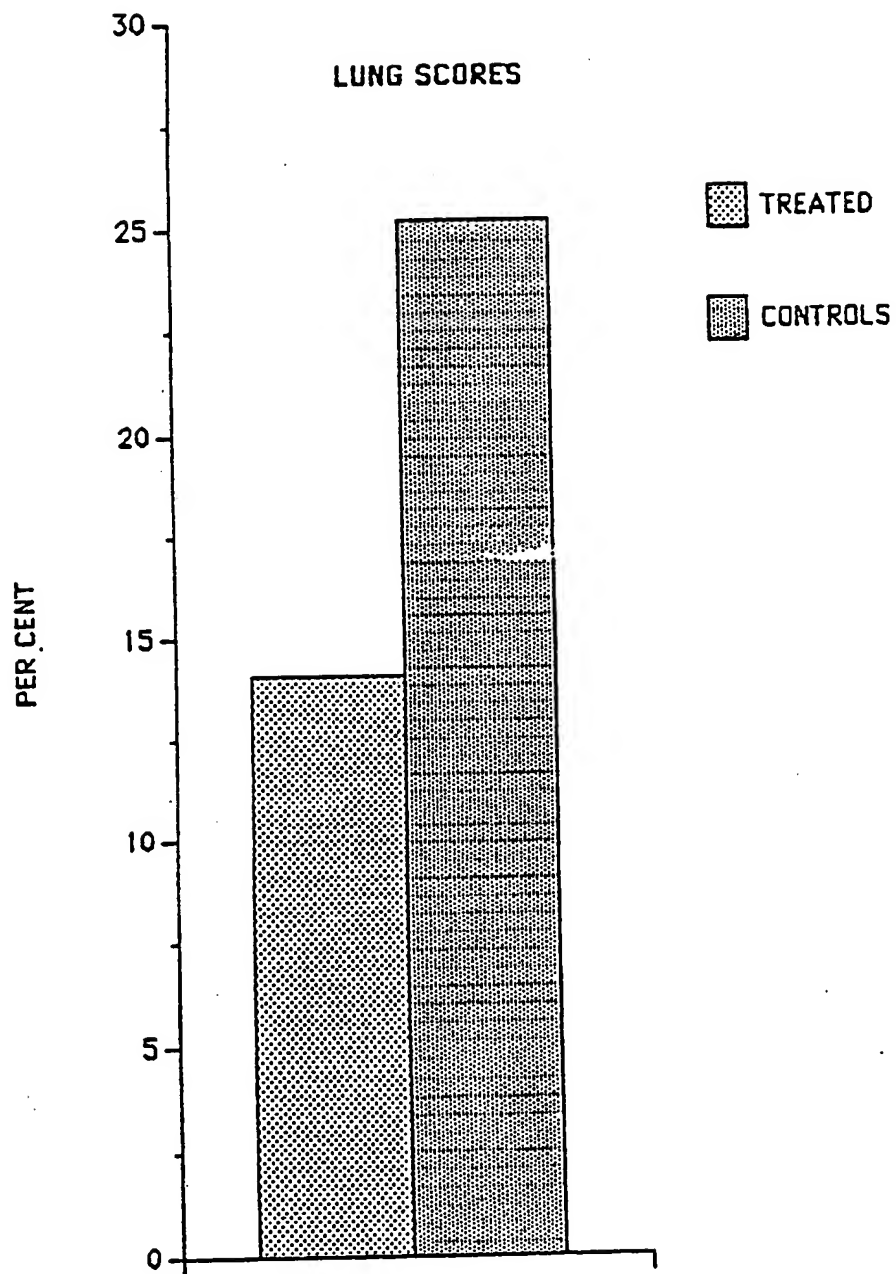




Figure 13

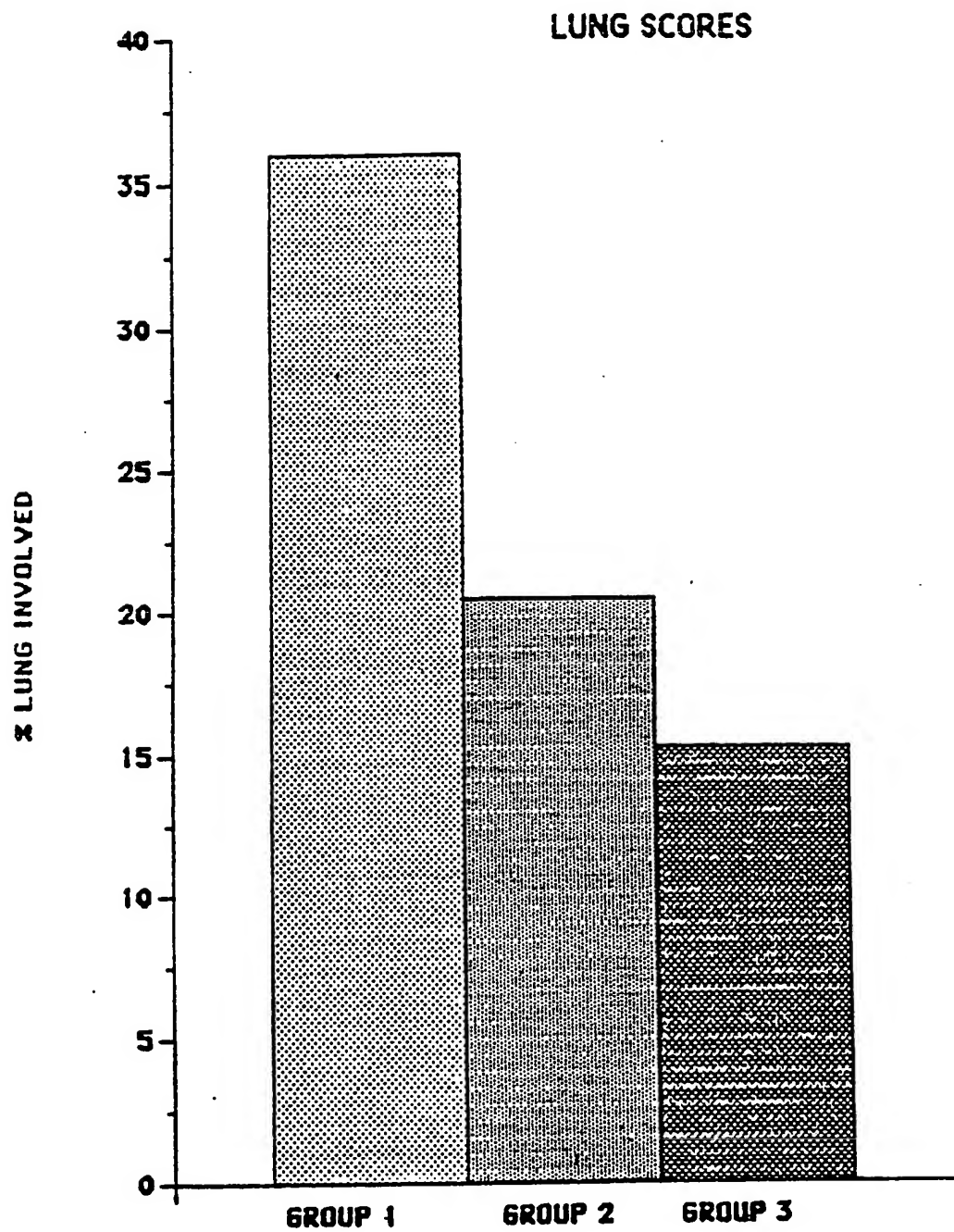


Figure 14

## DAILY MORBIDITY SCORES

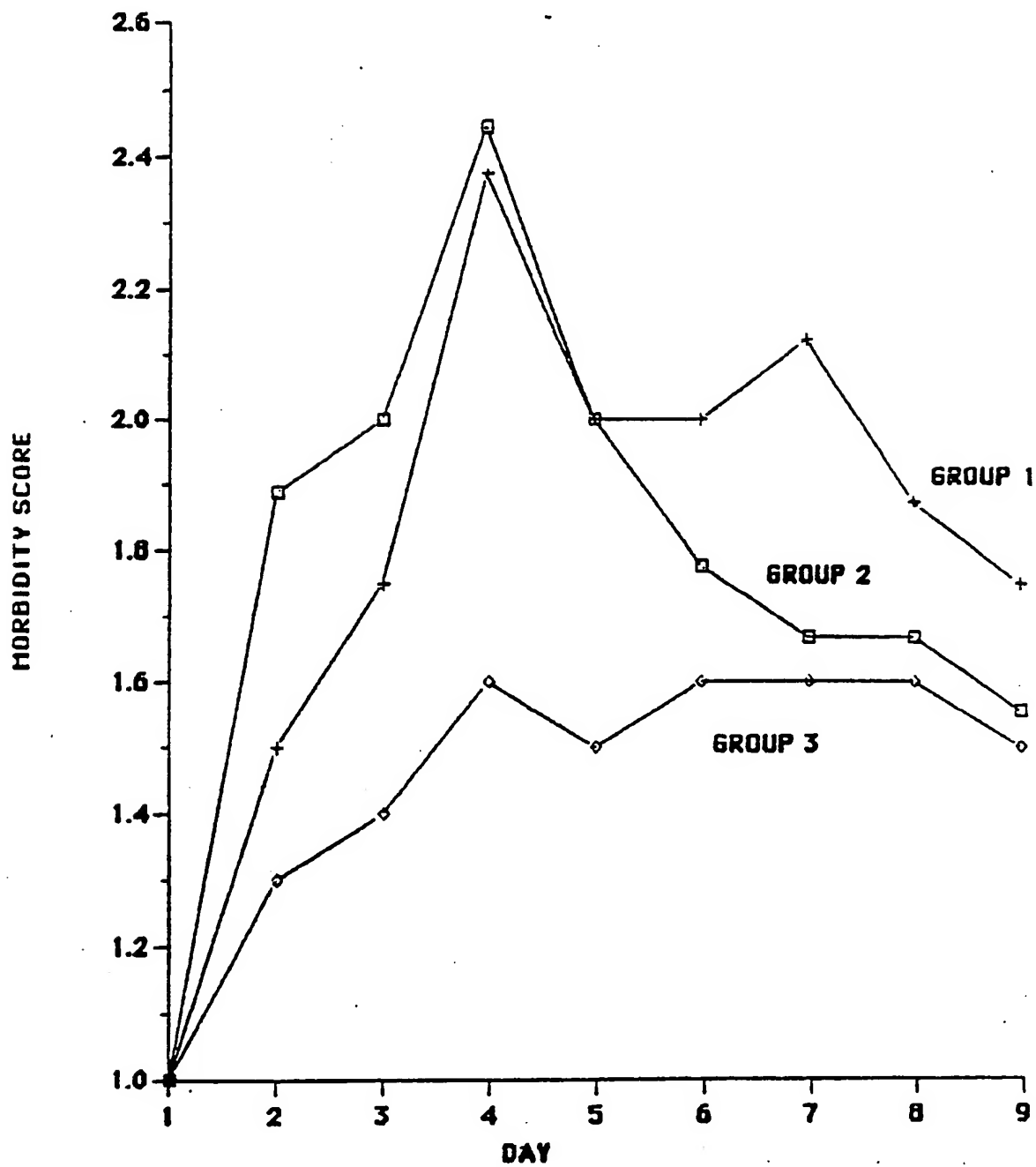


Figure 15

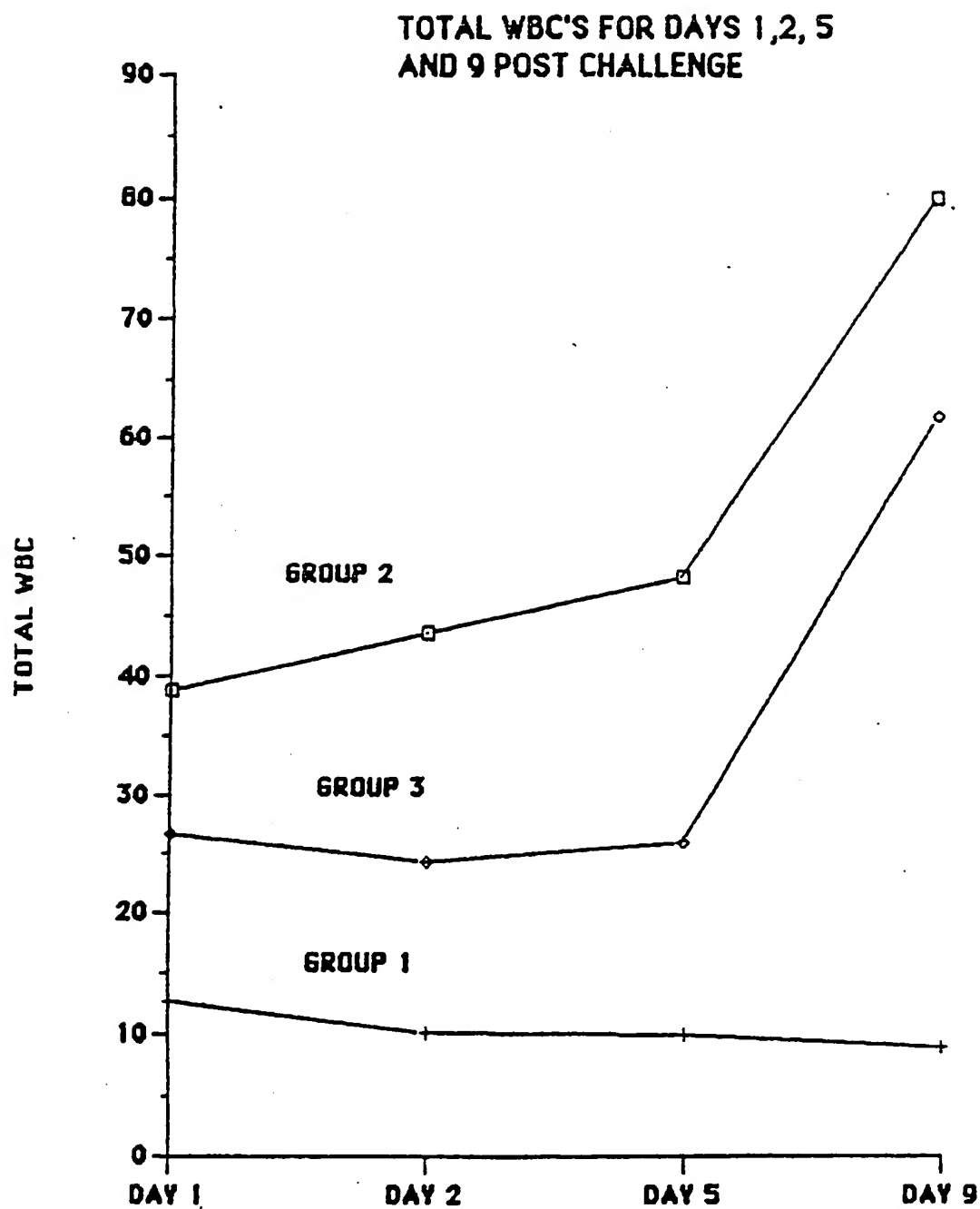


Figure 16

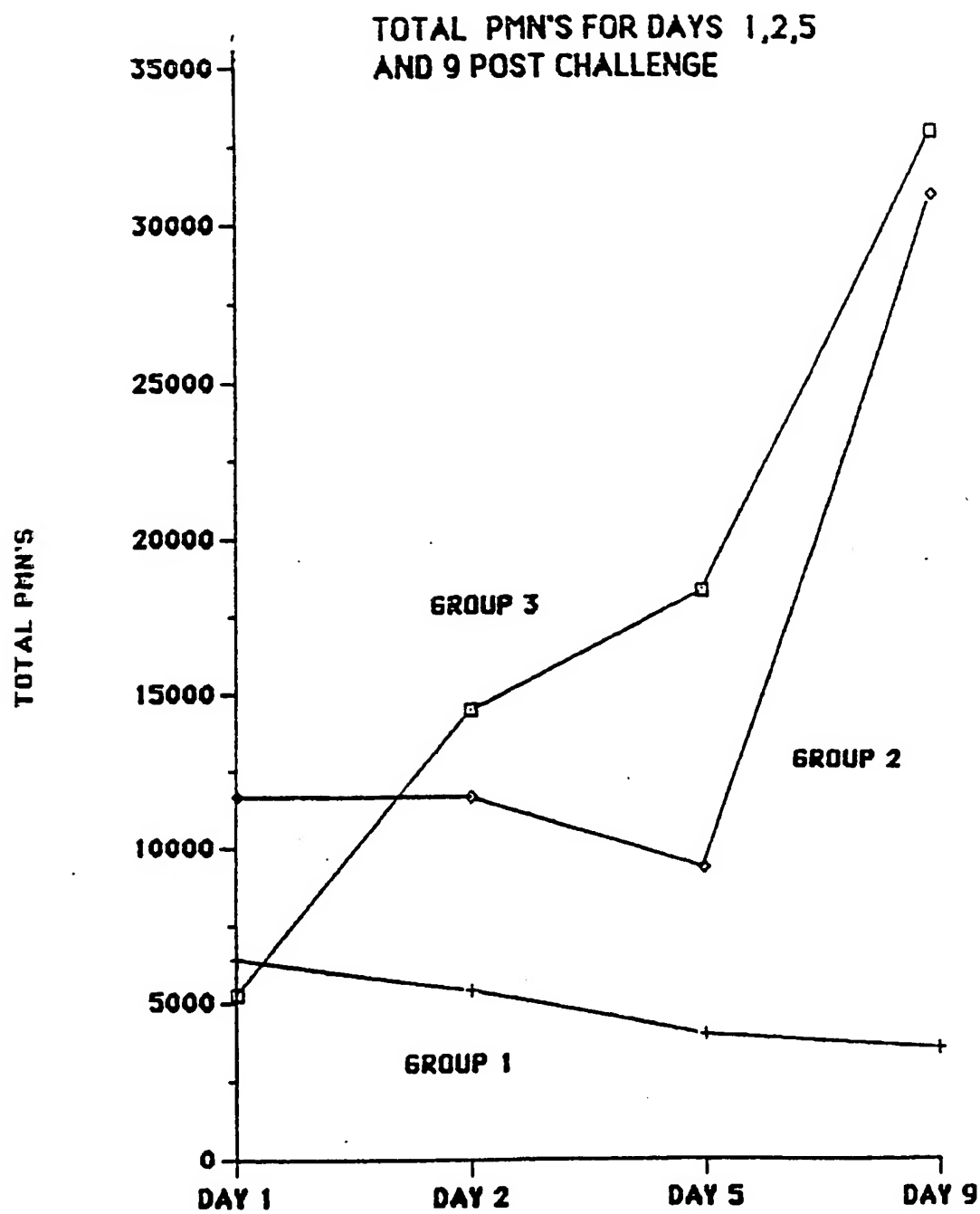
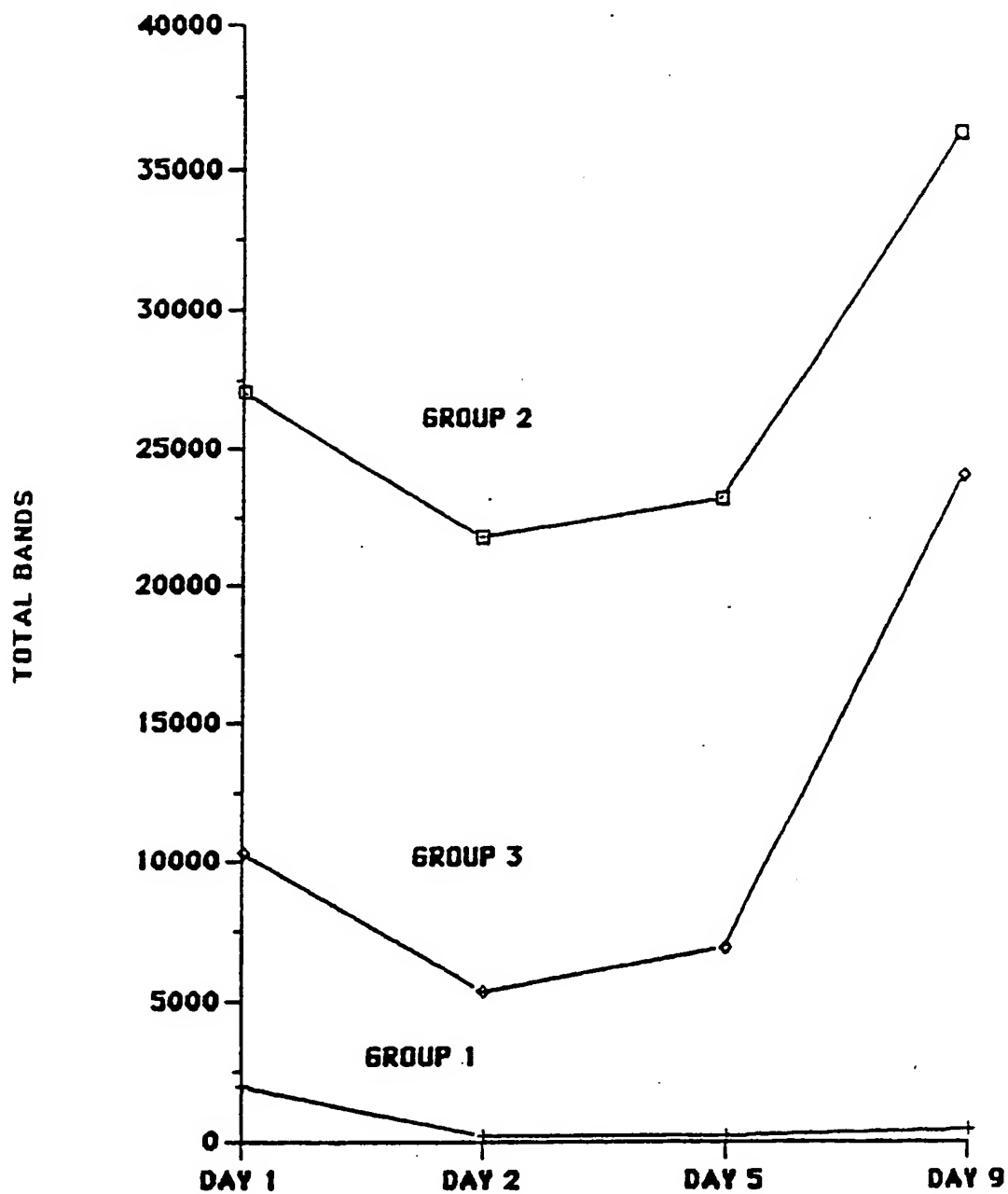
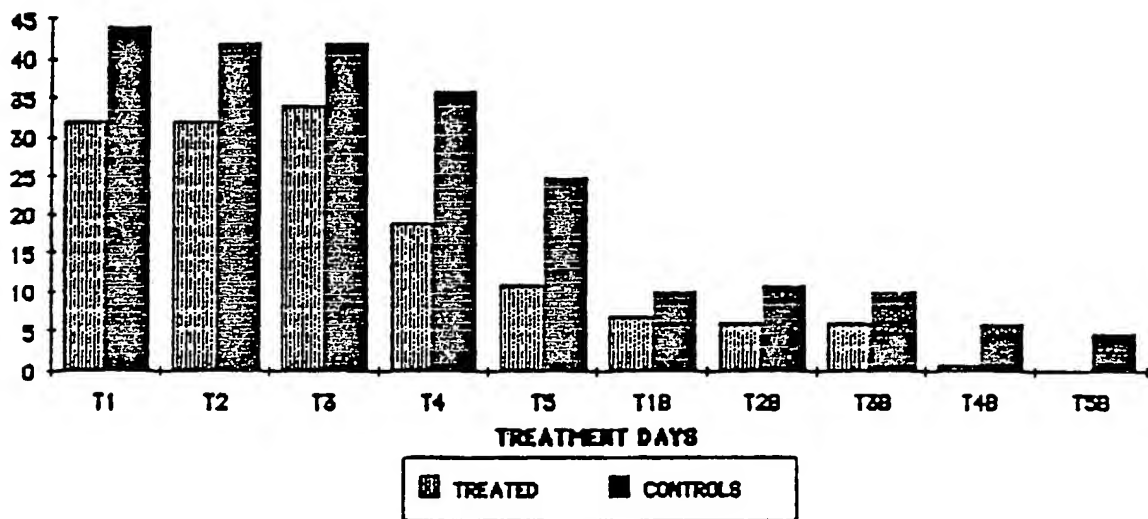


Figure 17

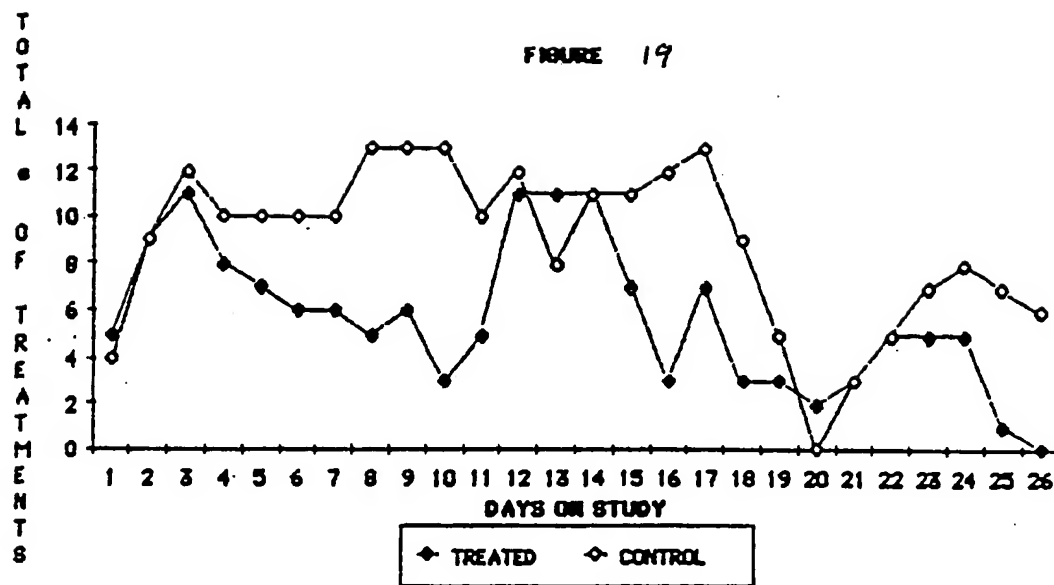
TOTAL BANDS FOR DAYS 1, 2, 5  
AND 9 POST CHALLENGE

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FIGURE 18



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FIGURE 20

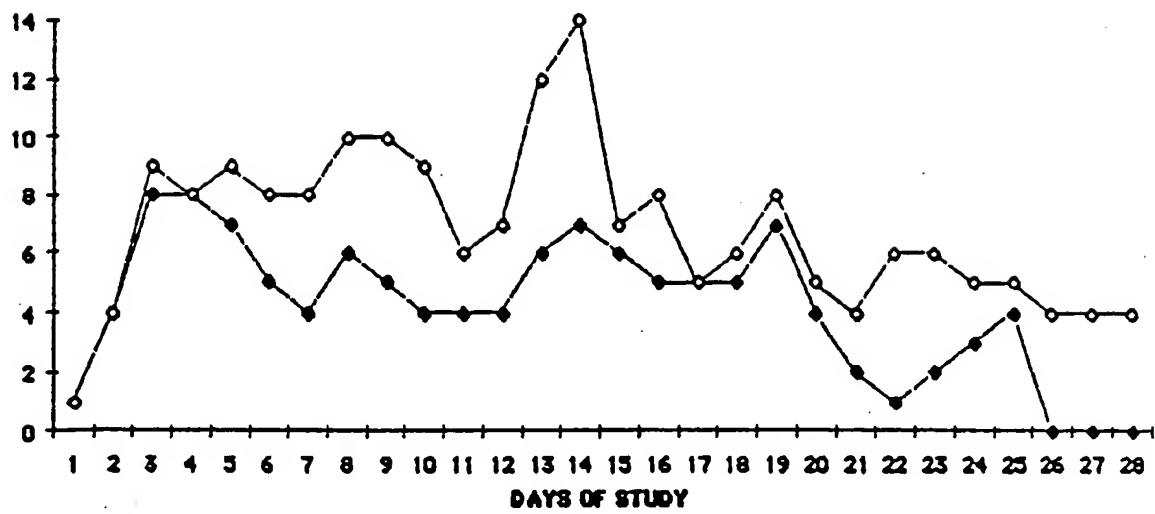




Figure 21  
COW 736  
Treatment Challenge

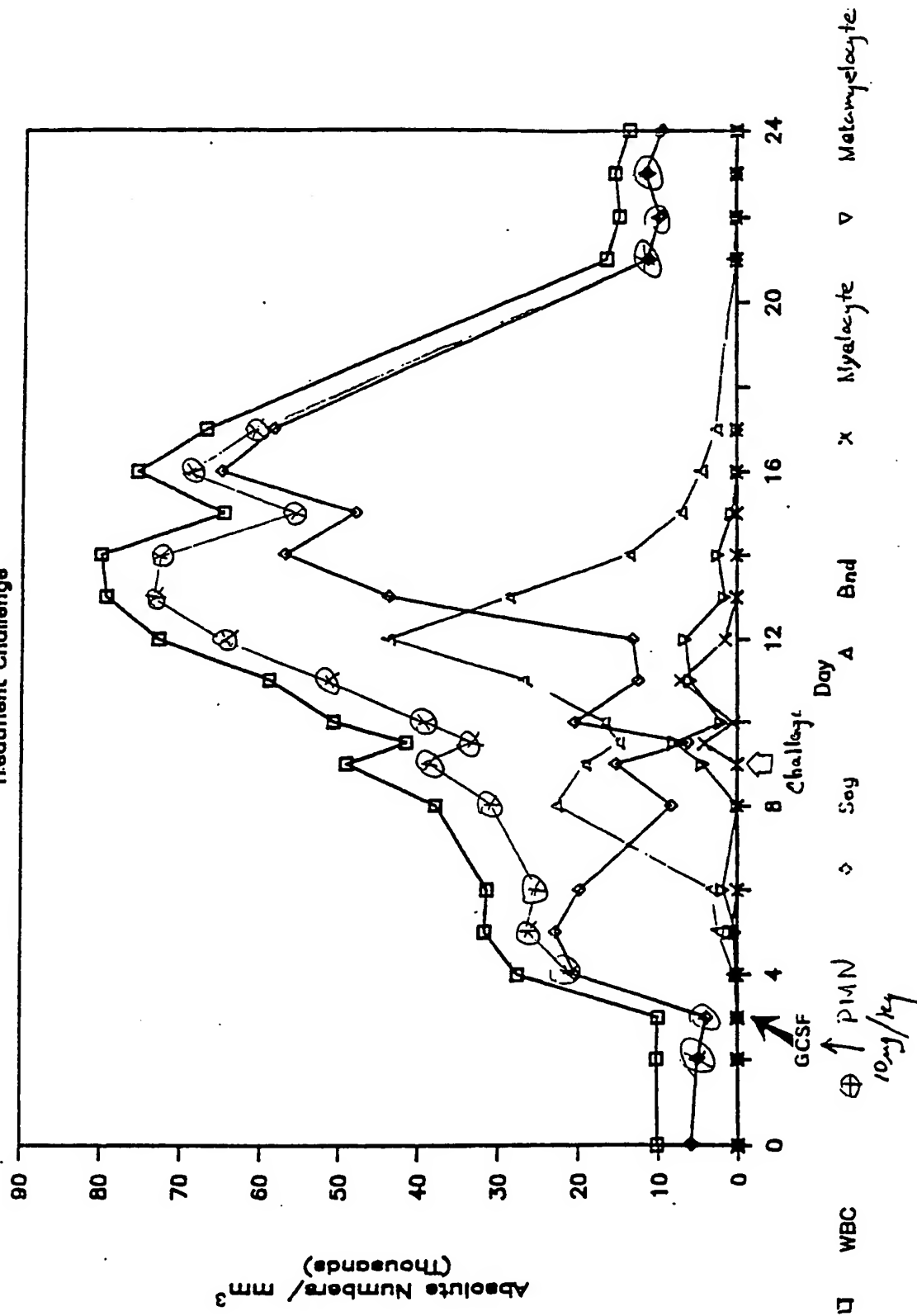


Figure 22  
Cow 293  
Treatment Control

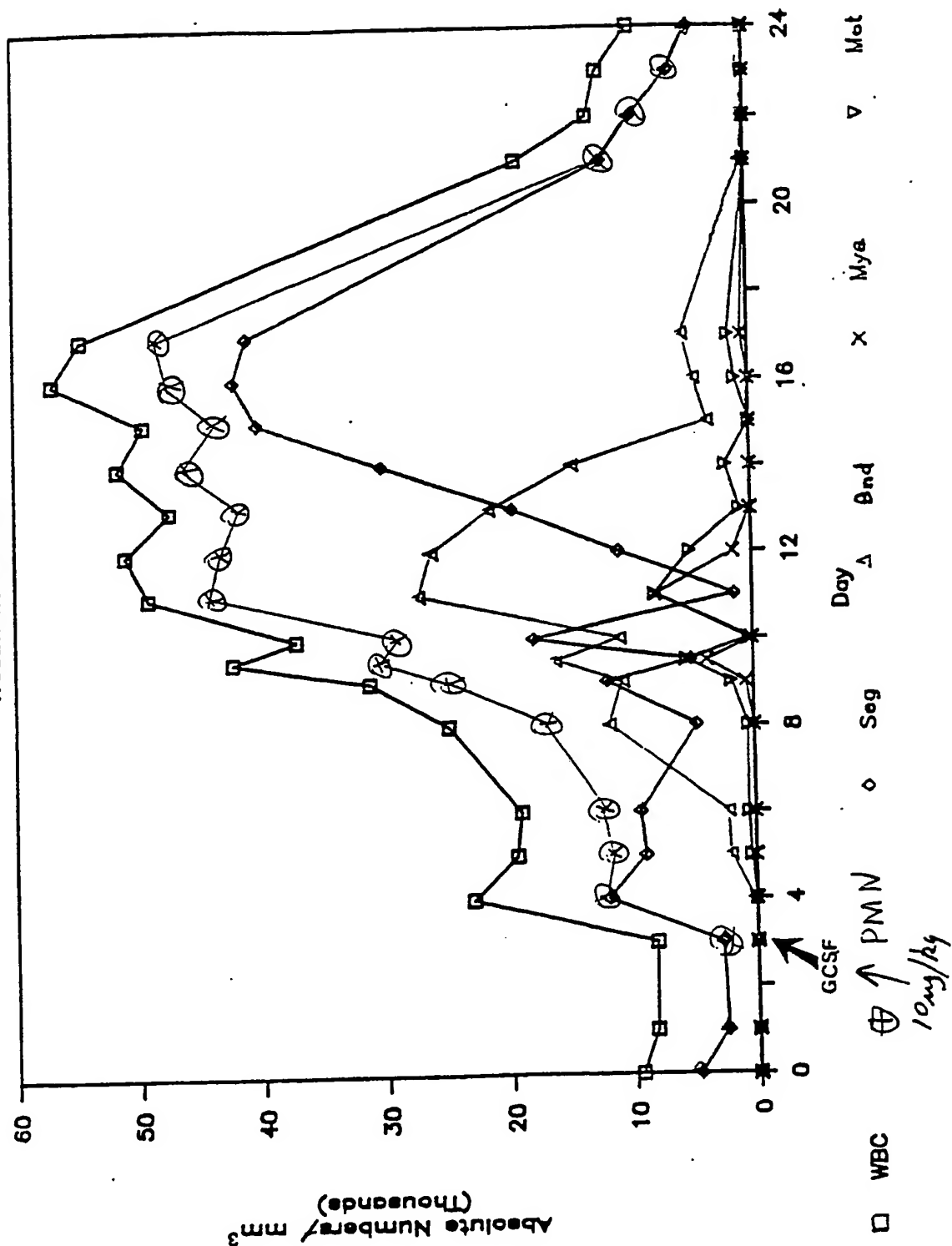
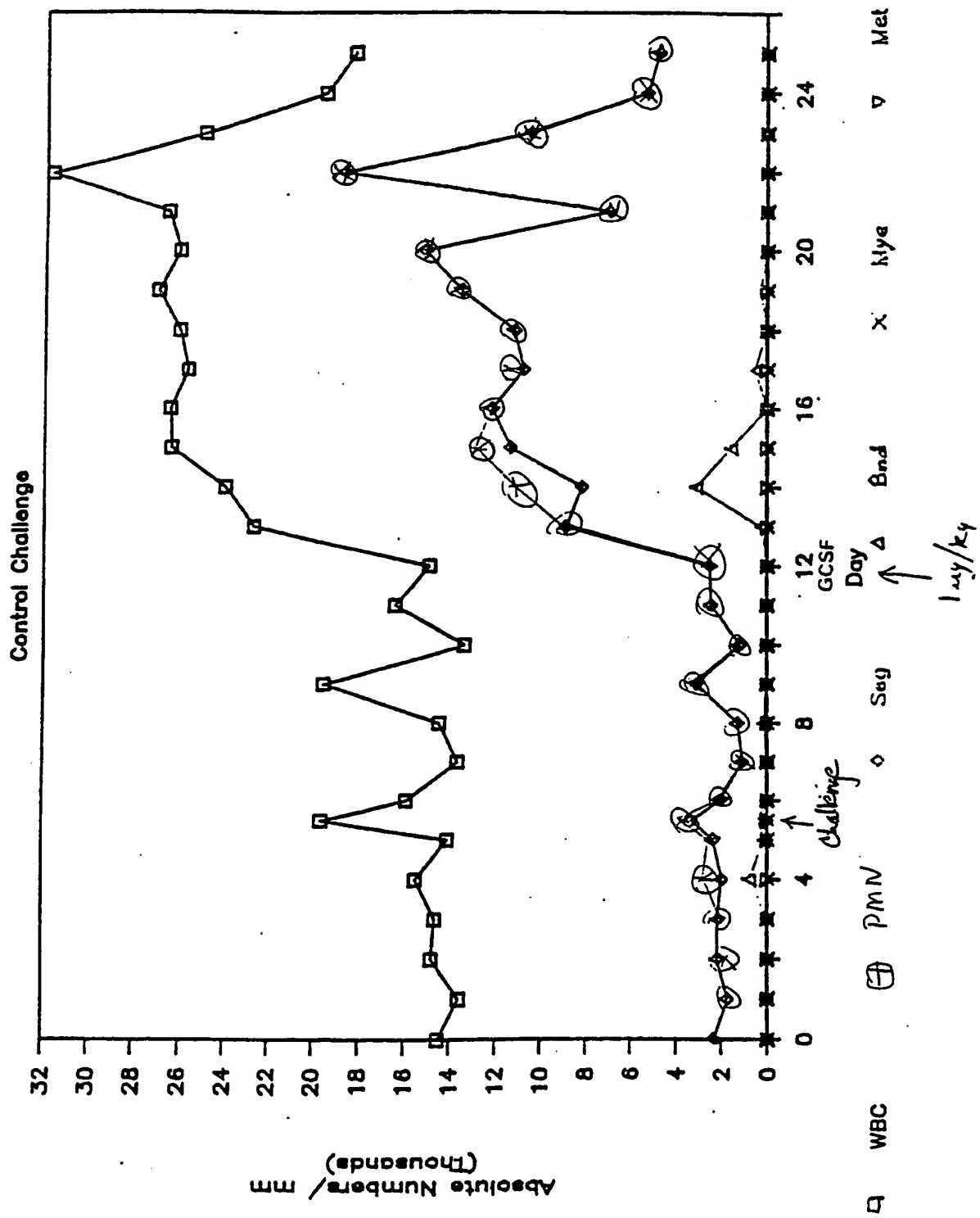


Figure 23  
COW 771



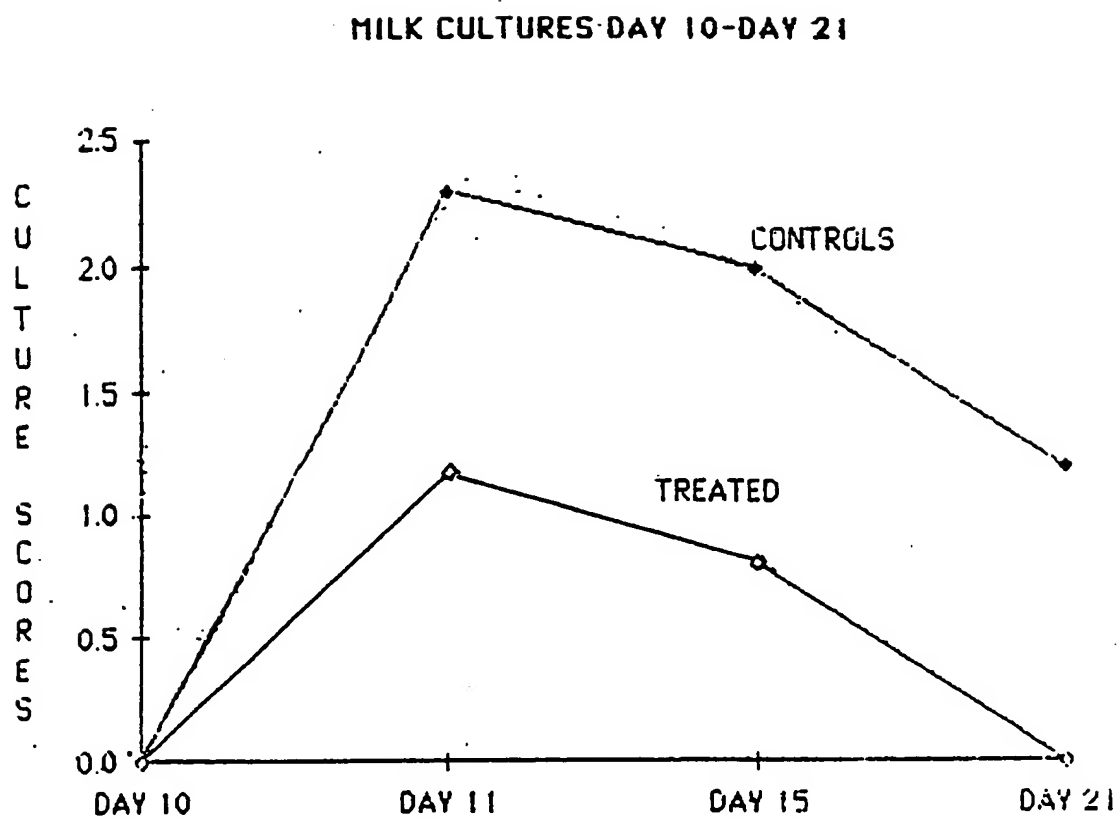
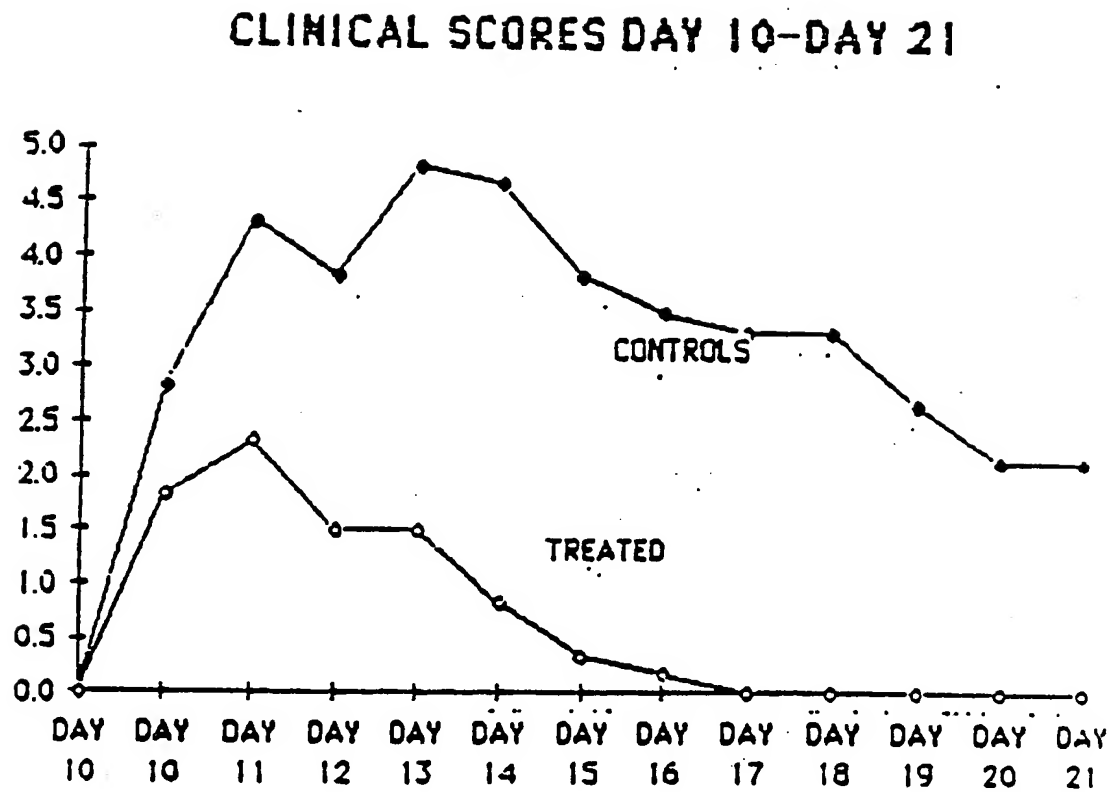


Figure 24



*Figure 25*

## CMT SCORES DAY 10-DAY 21

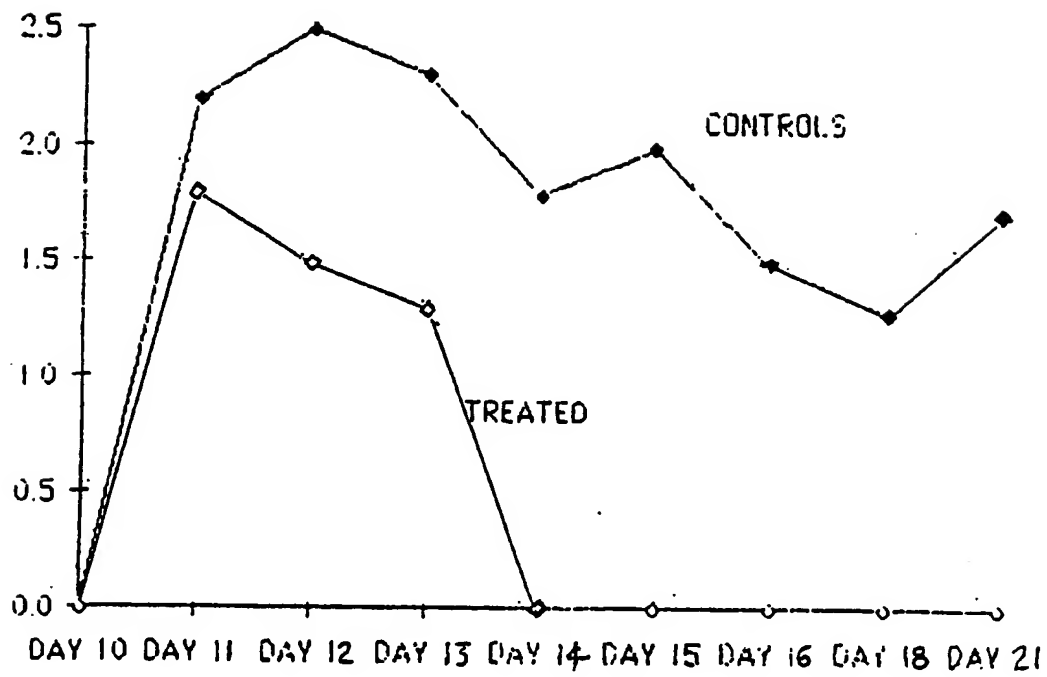


Figure 25

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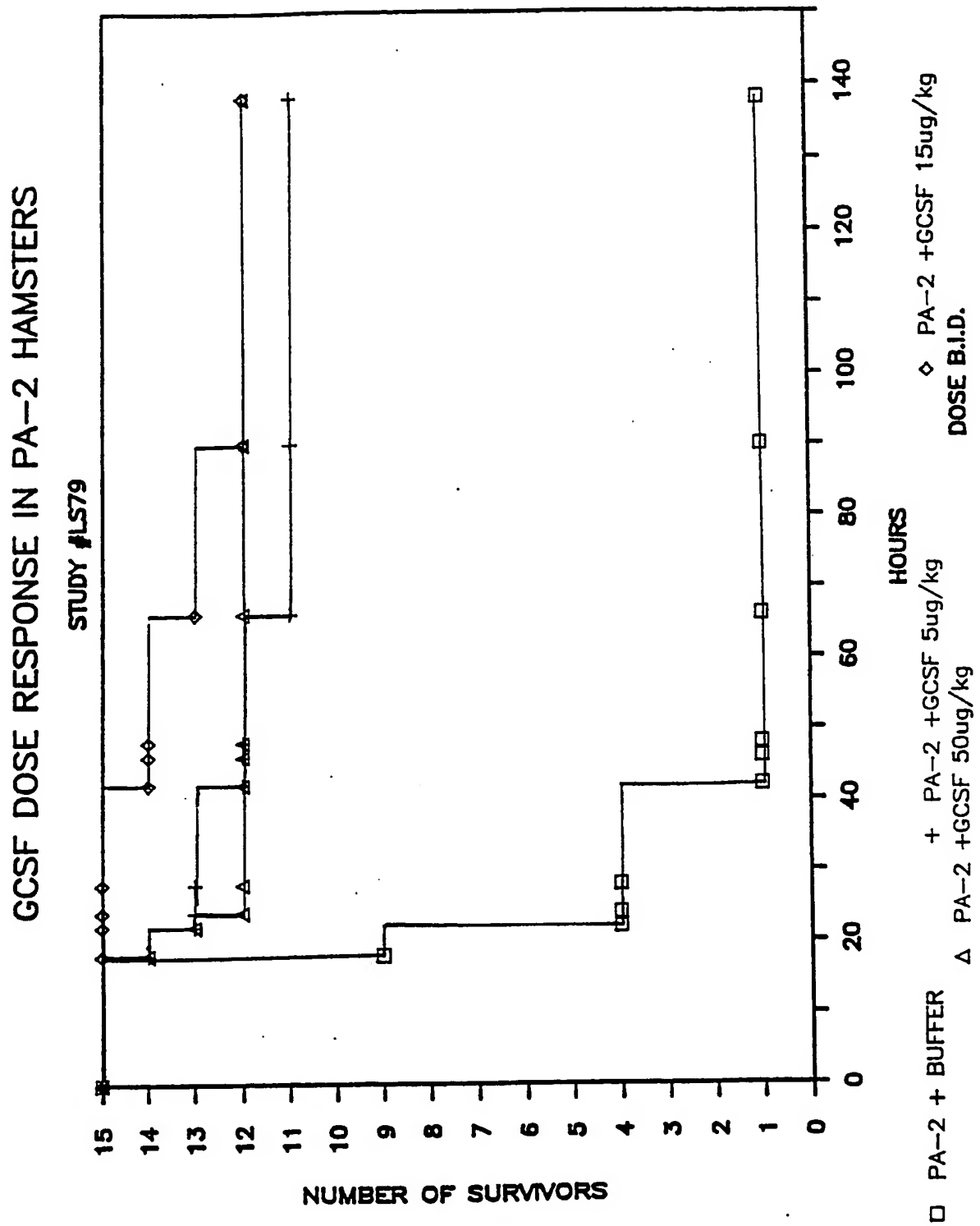


FIG. 27

## PA-2 HAMSTER PNEUMONIA GCSF DOSING

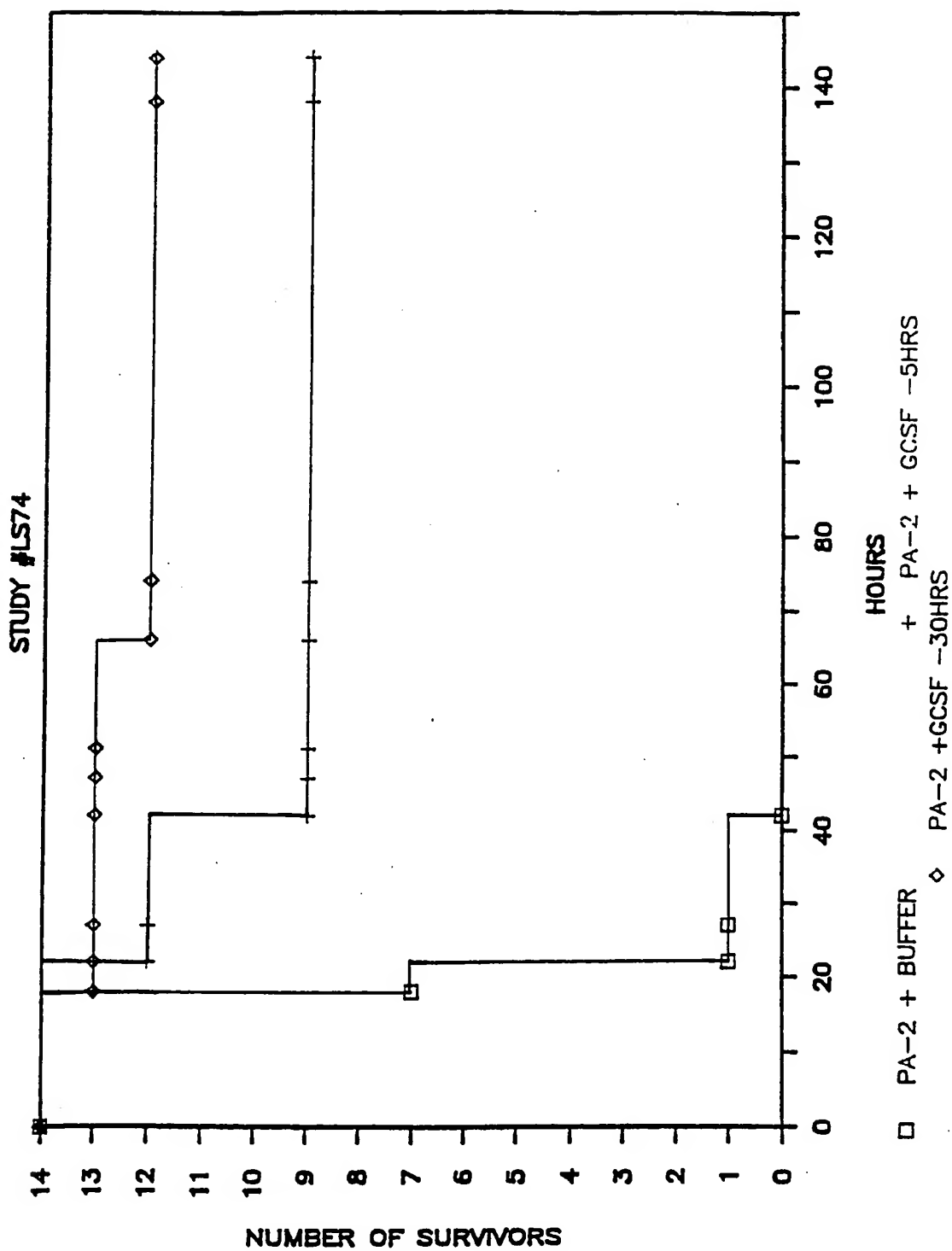


FIG. 28



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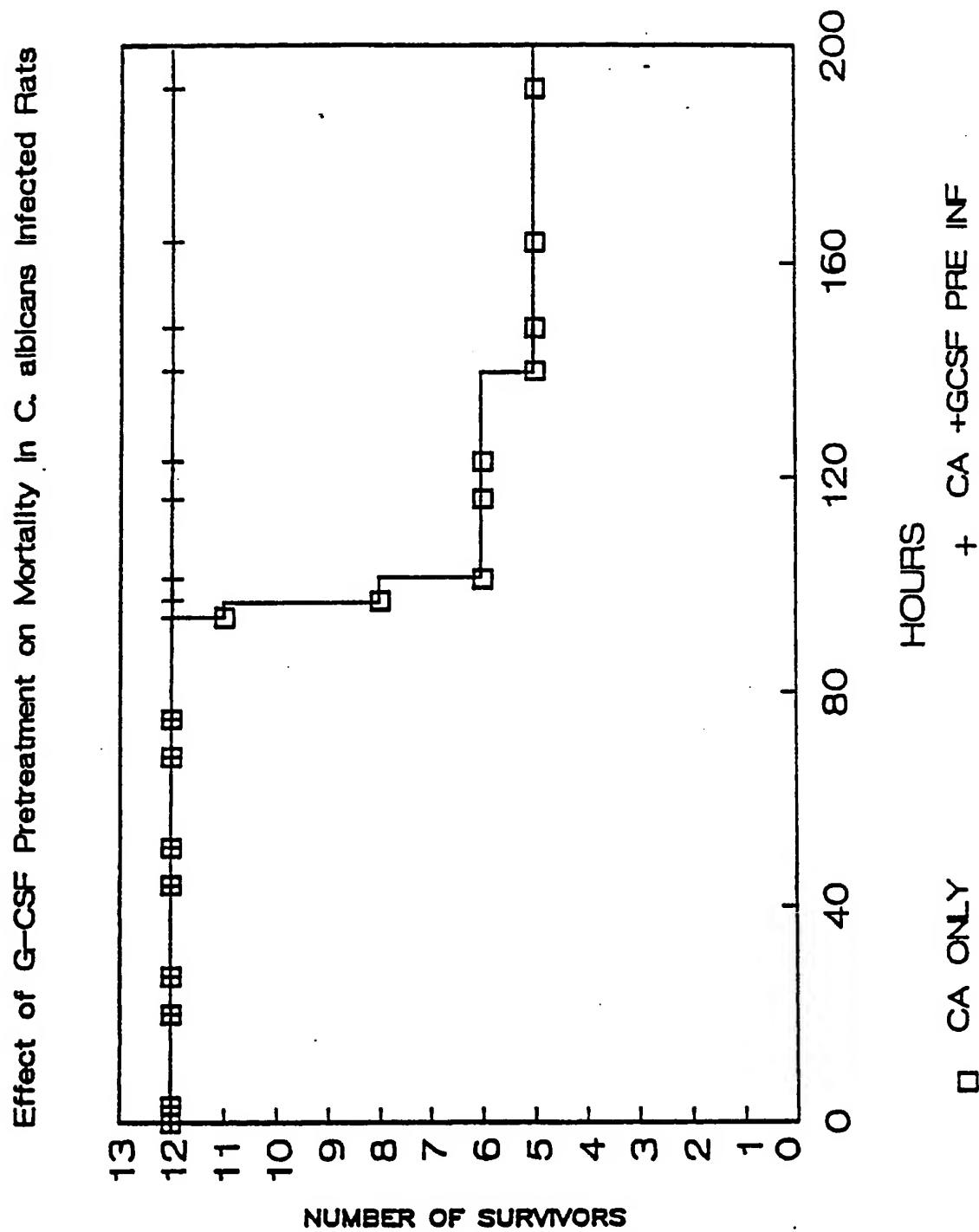
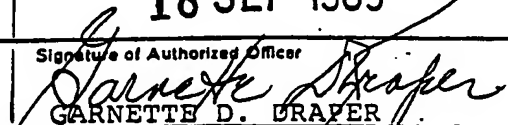


FIG. 29

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) * According to International Patent Classification (IPC) or to both National Classification and IPC INT CL <sup>4</sup> C07K 3/28,13/00; C07H 19/06; C12P 21/00,19/34; C12N 15/00; A61K 37/02 US CL 435/68,70,172.3,320,240.1; 536/27; 530/351,300,402,412; 514/2,8; 424/85.1		
<b>II. FIELDS SEARCHED</b> Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
US	435/68,70,172.1,172.3,320,240.1,240.2,243,253;536/27 530/300,351,402,412,416-419;514/2,8;424/85.1,85.2	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
Computer search on CAS and DIALOG; Files: CA, BIOSIS, 155,350,351; For: Bovine G-CSF and (recombinant, DNA, clone, purify, and treat/inhibit mastitis or shipping fever).		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> *		
Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
Y,P	US,A, 4,810,643, (Souza) 07 March 1989. See Claims.	1-64
Y	PCT, B, 86/04506, (ONO), 14 August 1986, (abstract), See abstract.	1-64
Y	The EMBO Journal, Vol. 5, Issued March 1986 "The Chromosomal gene structure of two m-RNAs for Human Granulocyte Colony-Stimulating Factor", (Nagata), pages 575, See pages 576, 578.	1-64
Y	Proc. Natl. Acad. Sci., Vol. 83, Issued October 1986, "Isolation and Characterization of the cDNA for Murine Granulocyte Colony- Stimulating Factor", (TSUCHIYA), pages 7633-37 see pages 7635-76.	
* Special categories of cited documents: <sup>10</sup> "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the International filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such docu- ments, such combination being obvious to a person skilled in the art. "A" document member of the same patent family		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search 18 August 1989 International Searching Authority ISA/US		Date of Mailing of this International Search Report 18 SEP 1989 Signature of Authorized Officer  GARNETTE D. CRAVER

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Methods in Enzymology, Vol. 116, Issued 1985, "Granulocyte Colony Stimulating Factor," (NICOLA), pages 600-619, See pages 605-614.	58-64
Y	Hoppe-Seyler's Z. Physiol. Chem., Vol. 363, Issued December 1982, "Isolation of A High Molecular Mass Granulocyte Colony-Stimulating Factor From Bovine Lung Conditioned Medium," (NEUMEIER), pages 1493-1500, See pages 1493-94.	1-64
.Y, P	Molecular Immunology, Vol. 25, Issued September, 1988 "Bovine GM-CSF: Molecular Cloning and Biological Activity of the Recombinant Protein," (MALISZEWSKI), pages 843-50, See page 849.	31-43, 52-57
Y	Infection and Immunity, Vol. 55, Issued 1987, "Protective Effect of Human Granulocyte Colony-Stimulating Factor on Microbial Infection in Neutropenic Mice," (MATSUMOTO), pages 2715-20, See page 2715.	31-43, 52-57

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE<sup>1</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers \_\_\_\_\_, because they relate to subject matter<sup>12</sup> not required to be searched by this Authority, namely:

2. ☐ Claim numbers \_\_\_\_\_, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out<sup>13</sup>, specifically:

3. ☐ Claim numbers \_\_\_\_\_, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING<sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows: Group I, claims 1-8, 11-12, 26-28, 30, 44-51, to b-G-CSF polypeptides, 530/351; Group II, claims 9-10, 13-25, 29 to DNA, cells and plasmids, 435/68+; Group III, claims 31-43, 52-57, to methods of treatment, 424/85.1; Group IV, claims 58-64, to methods of purifying b-G-CSF, 530/412.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ATTACHMENT TO FORM PCT/ISA 210, PART VI

## DETAILED REASON FOR HOLDING LACK OF UNITY OF INVENTION:

The invention as defined by Group I (claims 1-8, 11-12, 26-28, 30, 44-51) is drawn to polypeptides of bovine G-CSF, classified in class 530, subclass 351. Group II (claims 9-10, 13-25 and 29) is drawn to DNA, cell lines, plasmids and methods of producing the polypeptides), classified in class 435, subclass 68, 172.3, and 320. Group III (claims 31-43, 52-57) is drawn to methods of treatment with the bovine G-CSF, classified in 514 and 424, subclasses 8 and 85.1 respectively. Group IV (claims 58-64) is drawn to a method of purifying bovine G-CSF, classified in class 530, subclass 412.

PCT RULE 13.2 permits claims to a (one) product, a (one) method of making the product, and to a (one) method of using the product. The invention of Group II represents such a combination. However, the products of Group I, the method of Group III, and the process of preparation of Group IV constitute different products; different methods of use; and different methods of preparing the products from those of Group II. And there are no provisions in RULE 13.2 for claims covering Multiple products or processes.